

**Model socialite, problem pathogen:
the evolution and ecology of cooperation
in the bacterium *Pseudomonas aeruginosa***

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Thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh

2008

"The most important unanswered question in evolutionary biology, and more generally in the social sciences, is how cooperative behaviour evolved and can be maintained in human or other animal groups and societies."

- Lord Robert May, former Chief Scientific Adviser to HM Government, in his final presidential address to the Royal Society, 30 November 2005.

Abstract

In recent decades we have learned that cooperation is an important and pervasive feature of microbial life. This revelation raises exciting possibilities. On the one hand, we can now augment our understanding of how social phenomena evolve by using microbial model systems to test our theories. On the other hand, we can use concepts from social evolution to gain insight into the biology of the microbes we hope to control or kill.

In this thesis I explore both possibilities. First, I consider the theoretical problem of how and when microbial cooperation might be subject to frequency- and density-dependence. Formerly, vague theory and a scant, sometimes contradictory empirical literature made it unclear when such patterns could be expected. Here, I develop theory tailored to a microbial context, and in each case, I test key predictions from the theory in laboratory experiments, using as my model trait the production of siderophores by the bacterium *Pseudomonas aeruginosa*.

Secondly, I consider the ecological consequences of cooperator-cheat dynamics in the context of an infection. Specifically, I use experimental infections of diverse host models to investigate the role of two cooperative traits, siderophore production and quorum sensing, in the pathogenesis of *P. aeruginosa*. When a successful infection requires cooperation among pathogens, theory predicts that conflict among co-infecting strains can undermine cooperation and hence decrease virulence; whereas, in the absence of cooperation, conflict could lead to heightened exploitation and hence increased virulence. This exciting idea has received little empirical attention to date but here I address this using multiple pathogen strains, multiple social traits, and multiple model hosts.

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Declaration

I declare that I composed this thesis myself, although I acknowledge below the various contributions others have made to the body of work described herein.

In particular, my work on frequency dependence (chapter 2) and density dependence (chapter 3) was motivated by a grant proposal written by my supervisors, Stuart West and Ashleigh Griffin, along with our long-term collaborator, Angus Buckling (University of Oxford). The theory contained in these same chapters was developed primarily by Andy Gardner (University of Edinburgh) and Stuart West but was guided by our combined biological insight.

For several of the infection experiments described in chapters 4 and 5, I was assisted by Tiffany Taylor, an undergraduate student under my supervision. A subset of the results presented here formed the basis for her Honours dissertation. Steve Diggle (University of Nottingham) helped me with certain stages in the generation of mutant bacterial strains and shared some unpublished data which I included in Table 5.1.

This work has not been submitted for any degree or professional qualification except as specified.

Adin Ross-Gillespie, 29th November 2008

Publications

The following published paper has arisen from this thesis and is included in the appendix.

A2: Ross-Gillespie, A., Gardner, A., West, S.A. and A. S. Griffin. 2007. Frequency dependence and cooperation: theory and a test with bacteria. *American Naturalist* 170:331-342.

During the course of my PhD I published two other papers on topics unrelated to the subject of this thesis, but which nonetheless pertain to my overarching research interest – the evolution of cooperation. These appear as appendices A3 and A4.

A3: Ross-Gillespie, A., O'Riain, M. J. and L. F. Keller. 2007. Viral epizootic reveals inbreeding depression in a habitually inbreeding mammal. *Evolution* 61:2268-2273.

A4: Ross-Gillespie, A. and A. S. Griffin. 2007. Quick Guide: Meerkats. *Current Biology* 17:442-443.

I contributed to two further papers that do not constitute chapters of my thesis. The first of these is first-authored by a collaborator of ours, Kendra Rumbaugh (Texas Tech University) and is conceptually closely related to the work I present in chapter 5. I contributed to this study by engineering knockout strains and helping with data analysis and manuscript preparation. In the second paper, first-authored by Max Burton, I helped to plan and conduct the research, and later contributed to the manuscript.

Rumbaugh, K. P., Diggle, S. P., Watters, C. M., Ross-Gillespie, A., Griffin, A. S. and S. A. West. Quorum sensing and the social evolution of bacterial virulence. *Current Biology*. *Submitted*.

Burton-Chellew, M., Ross-Gillespie, A. and S. A. West. Cooperation in humans: competition between groups and self-deception. *Evolution and Human Behaviour*. *Submitted*.

Acknowledgements

The work presented in this thesis benefited from many people's comments, discussion, collaboration and encouragement.

First and foremost, I thank my supervisors, Stuart West and Ashleigh Griffin, for their guidance, support and inspiration throughout. My special thanks go also to Andy Gardner and Nick Colegrave, my stand-in supervisors while Stuart and Ashleigh were away on sabbatical, and to our collaborators, Steve Diggle and Angus Buckling for their advice and assistance.

Next, I thank the members of my research (meta)group for their encouragement, support, suggestions and innumerable wee favours, especially Rolf Kümmerli, Alex Hall and Natalie Jiricny, but also Max Burton, Tabitha Innocent, Tiffany Taylor, Gavin Ballantyne, Suzanne Clarke, Piet van den Berg, Ben Evans, João Alpendrinha, Sarah Reece, Bart Pannebakker, Aleta Graham, Dave Shuker and Jun Abe.

I am grateful also to my many other friends at the University of Edinburgh who over the years have patiently endured my highs and lows and have kept me sane and entertained. In particular, I thank Christèle Gonneau, Pedro Vale, Adel Heenan, Juan-Carlos Ruiz Guajardo, Laura Ross, Ellie Barnwell, Helen Senn, Tom Reed, Antoine Claessens, Matt Robinson, Sarah Hall, Anjie Harris and Laura Kelley. More generally, I extend my heartfelt salute to all my colleagues at the Ashworth Laboratories. It has been an honour and a pleasure to work among folks of such brilliance and bonhomie.

My studies were funded by a scholarship from the School of Biological Sciences at the University of Edinburgh, with additional funding from the UK government's Overseas Research Students Awards Scheme. I gratefully acknowledge this support.

Finally, I thank my parents, Trevor and Shirley, who bemusedly tolerated my aversion to those other more sensible, stable careers, and supported me all the same.

1. Sociomicrobiology: an introduction

The work in this thesis represents a fusion of social evolution theory and microbiology. This area of research, dubbed ‘sociomicrobiology’, is a fledgling field, but one that is growing rapidly as scientists from either background increasingly recognise the potential, and indeed the need, for synergy between these two historically disparate disciplines (Velicer 2003; West et al. 2006; Dethlefsen et al. 2007; West et al. 2007a). In the following sections of this chapter, I briefly review the significance of this union, discussing, in turn what microbiology has to offer social evolution (section 1.1), and then what social evolution has to offer microbiology (section 1.2). I provide only a brief synopsis here because each later chapter contains its own independent introduction with relevant background. In the final section of this chapter (1.3) I provide a plan for the rest of this thesis, outlining how my own research addresses the challenges in this field.

1.1. How can microbiology help social evolution?

Social evolution concerns the study of ‘social’ behaviours[†], which are those that affect not only a focal individual but others too. Such behaviours can be classified on the basis of their direct fitness consequences for both the actor and the recipient(s) (Table 1.1; Hamilton 1964; Trivers 1985; West et al 2007c).

Table 1.1. Social behaviours classified according to their direct fitness consequences.

Effect on actor		Effect on recipient	
		+	-
+	+	Mutual benefit	Selfishness
-	-	Altruism	Spite
		COOPERATION	

[†] ‘Behaviour’, as it is used here, can be regarded as synonymous with ‘trait’ or ‘adaptation’. It refers to any action undertaken by-, or any morphological structure associated with, a given biological entity. Thus, the transmission of a selfish genetic element, the secretion of proteins from a cell, a plant’s tolerance for rhizobial colonization of its root nodules, or the territorial contest between two baboons can all be thought of as ‘behaviours’.

Behaviours beneficial to the actor can be ‘selfish’ if the recipient suffers costs, or ‘mutually beneficial’ if the recipient also benefits from the interaction[‡]. Behaviours costly to the actor could be ‘altruistic’ if beneficial to the recipient, or ‘spiteful’ if costly to both parties (see Gardner and West 2004 for further discussion on the evolution of spite). Altruism and mutual benefit together comprise ‘cooperative’ behaviours – those which provide benefit to others (and which have evolved at least partly because of this benefit). In practice, it can be difficult to distinguish between these two, because an action that incurs a cost to the actor in the short term (appearing to be an altruistic act) could result in some deferred direct benefit at a later time point (and thus be mutually beneficial). Also, mutual benefits are typically asymmetrical, so – at least when interacting parties are part of the same competitive pool (i.e. local, intraspecific interactions) – such behaviours will usually ultimately tend towards either selfishness (if the actor derives greater benefit) or altruism (if the recipient derives greater benefits from the interaction).

Cooperation is widespread in nature and has underpinned all the major transitions between levels of biological complexity (Maynard Smith and Szathmàry 1995). Despite its ubiquity however, it presents a serious evolutionary conundrum: against a backdrop of Darwinian “survival of the fittest”, how can cooperation evolve if it increases the fitness of a recipient relatively more than it does the fitness of the actor? Surely cheats – individuals which do not cooperate but are able to exploit the cooperation of others – would gain a competitive advantage and thus be able to invade and out-compete a population of cooperators? This problem is well recognized in the fields of economics and sociology too (e.g. the ‘tragedy of the commons’; Hardin 1968).

Given the ubiquity of cooperation in the natural world, solutions to the problem of cooperation must obviously exist. Accordingly, much effort has gone into finding biologically plausible explanations for the evolution of cooperation, particularly in the wake of William D. Hamilton’s seminal insights of the 1960s, when he recognized that an actor’s behaviour could influence its fitness indirectly too via its

[‡] Note that the term ‘mutualism’ is historically reserved for instances of interspecific cooperation (West et al. 2007c).

impact on other individuals, and that the direction and magnitude of this influence depended on the genetic correlation between the actor and the recipient (kin selection; Hamilton 1964).

A profusion of models and mechanisms has since been proposed, involving either direct or indirect fitness benefits, or some combination of the two (Figure 1.1). In recent years, significant progress has been made towards bringing this vast body of work together into a common, comprehensive conceptual framework (Sachs et al. 2004; Foster and Wenseleers 2006; Lehmann and Keller 2006; Bergmüller et al. 2007; West et al. 2007b; Bshary and Bergmüller 2008; Gardner and Foster 2008). There has also been progress towards resolving the considerable semantic confusion that has dogged this field (West et al. 2007c) and in developing and promoting a versatile, powerful approach with which to mathematically formalize and link these various concepts (inclusive fitness theory; Hamilton 1964; Taylor and Frank 1996; Frank 1998; Grafen 2006; Lehmann and Keller 2006; Grafen 2007; Lehmann et al. 2007; Taylor et al. 2007)

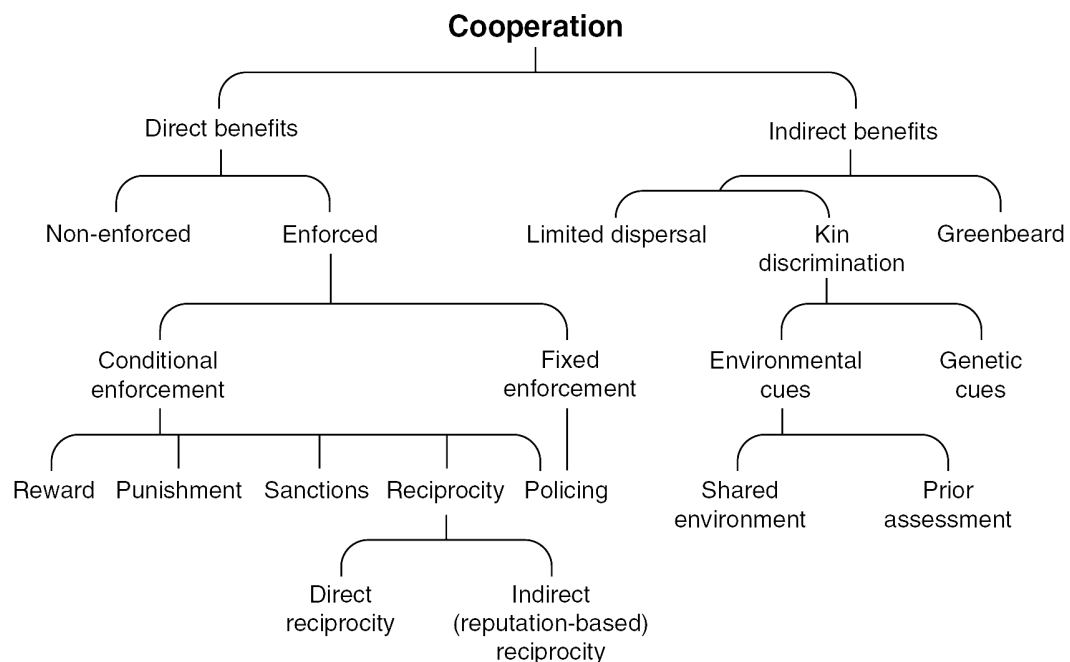


Figure 1.1. A classification scheme for the various mechanisms whereby cooperation can evolve. Although direct benefits are principally important for mutually beneficial cooperation and indirect benefits for altruism, these mechanisms are not mutually exclusive and may often operate synergistically to facilitate the evolution of cooperation. This figure is redrawn from West et al. (2007b), which discusses these mechanisms in greater detail.

Theoretical advances in the field of social evolution have outpaced empirical work. The majority of this theory was devised to explain known behaviours in animals such as insects, birds and mammals, and although the general concepts should be applicable across all biological systems, there has been little empirical testing of these ideas outside of these select taxa (West et al 2007a). Moreover, some aspects of theory have been overlooked by empiricists because to test them experimentally would require manipulations that are impractical or impossible in traditional systems. For example, to experimentally test the idea that local competition could counterbalance kin selection for cooperation (West et al. 2001; Griffin and West 2002) requires that relatedness be varied independently of the scale of competition - something not easily achieved in traditional study systems (but see Griffin et al. 2004).

This is where microbiology comes in. Microbial systems are uniquely amenable to experimentation. Microbes can be cultured under laboratory conditions, quickly, in large population sizes and with high levels of replication. The fitnesses of different genotypes can be easily monitored across multiple generations and the course of their evolution can be influenced by artificially imposed selection regimes, tracked in real time, and even halted whenever desired by simply popping them in the freezer! Moreover, in the case of the better studied microbial model organisms, much is already known about the genetic details underlying a given trait, such that genetic mutants for a trait of interest can be relatively easily generated using targeted mutagenesis, or failing that, non-targeted mutagenesis using radiation or chemical treatments (Foster et al. 2007).

Microbial systems, therefore, offer social evolution excellent opportunities to confirm the generality of existing theory and test previously untested (or poorly tested) predictions. Their unique biology can also provide novel challenges for which new theory is appropriate, such as quorum sensing (Brown and Johnstone 2001) or frequency-dependent cooperation (chapter 2). Finally, because many microbes are medically or economically important, they present a rare opportunity for scientists working on social evolution to apply their skills and experience to situations where

greater biological insight could lead to a more productive or sustainable industrial process, or significant benefits to our health and wellbeing and that of the organisms we care about (Nesse and Stearns 2008).

1.2. How can social evolution help microbiology?

The classic view of microbes as simple organisms that live out essentially solitary existences has been dramatically overturned in the past few decades with the discovery that, in fact, microbes are intensely social organisms. Indeed, microbes exhibit a striking array of social behaviours (Shapiro 1998; Crespi 2001; West et al. 2007a; Davidson and Surette 2008). Numerous microbial systems have now been described in which individuals variously communicate and cooperate to disperse, acquire food and counter abiotic or biotic threats. Such phenomena have understandably attracted enormous interest from microbiologists, who have set to work deploying, usually to great effect, an impressive array of modern technology, to uncover the molecular and biochemical details underlying each system.

There has however, been considerable confusion in the microbiological literature when it comes to interpreting the ultimate, evolutionary forces that facilitate and govern these phenomena. The fact that cooperation among individual microbes can lead to benefits at the population level does not itself provide an evolutionary stable solution to the problem of cooperation (West et al. 2007a), yet the microbiological literature is replete with claims that cooperative behaviours have evolved ‘for the good of the group’ (e.g. Shapiro 1998). I provide a few examples below:

- Biofilms – intricate surface-attached communities – represent a common mode of growth for many microbes (Kolter and Greenberg 2006). Growth in biofilms confers obvious benefits at a group level. These matrix-enclosed ‘slime cities’ (Watnick and Kolter 2000) provide a stable, protective environment and act as a nidus for the dissemination of dispersal units (Hall-Stoodley and Stoodley 2005). Observing that biofilm cultures are often characterized by greater genetic and phenotypic diversity than planktonic

cultures (Stewart and Franklin 2008), and that genetically diverse biofilms have a greater likelihood of persisting under changeable environmental conditions, Boles et al. (2004) postulate that this “... *self-generated diversity in biofilms provides a form of biological insurance that can safeguard the community in the face of adverse conditions.*” Chia et al. (2008) share this view: “*The different types of cells rely on each other, so that their mutual interactions lead to the overall success of the group.*” Watnick and Kolter (2000) extend this thinking to multispecies biofilms: “*We liken the multispecies bacterial biofilm to a city where bacteria settle selectively, limit settlements of new bacteria, store energy in exopolysaccharide, and transfer genetic material horizontally, all for the good of the many.*”

- In microbial populations facing starvation or stress it is often observed that a subpopulation of specialized “persister” cells develops (Lewis 2007). These are metabolically quiescent but hardy phenotypes with greatly improved prospects for survival under stressful conditions. Palkova (2004) interprets this phenomenon as follows: “... *multicellular existence allows individual cells to differentiate and acquire specific properties, such as forming resistant spores, which benefit the whole population.*”
- Apoptosis (cell suicide) routinely occurs within metazoan tissues but also in microbes (Bayles 2007; Büttner et al. 2007), where its function has attracted much speculation: “... *in yeast colonies, part of the population sacrifices itself for the benefit of the rest of the population*” (Palkova and Vachova 2006)
- Cell-to-cell signaling (quorum sensing), thought to operate in most microbial taxa (Bassler and Losick 2006), is another phenomenon of major biological significance, and it too is widely assumed to have evolved because of the benefits it confers at a population-level. Lyon (2007) suggests that this situation has been further exacerbated by the use of loaded anthropomorphisms when referring to this phenomenon, such as “language” (Bassler 2002; Sperandio et al. 2003), “talking” (Kaiser and Losick 1993; Winzer et al. 2002), “listening” (Fuqua and Greenberg 1998), “eavesdropping” (Von Bodman et al. 2003; Wagner et al. 2006), and even “linguistic communication” (Ben Jacob et al. 2004).

The past 45 years of social evolution research have shown us that this concept of traits evolving “for the good of the group” is misguided (West et al. 2008; Gardner and Grafen, *in press*). On the contrary, one of the most important principles to have emerged from these decades of social evolution research is that selection acts to maximize the inclusive fitness of individuals. Certainly, the relative success of a group in competition with other groups can feed back into the inclusive fitness of individuals that comprise that group (multi-level selection), and in this sense, group-selected benefits can influence the course of evolution; however, only under certain restrictive conditions – such as when groups are wholly clonal, or competition is completely suppressed – will the interests of individuals and groups may be sufficiently aligned for selection to appear to act at the level of the group. In general, this is considered a weak and largely unimportant force in evolution (Maynard Smith 1976; West et al. 2006).

A major problem with the “for the good of the group” paradigm is that it tends to overlook the inevitable potential for conflict of interest within groups. Wherever there is cooperation, so too we would expect conflict. Conflict is especially likely within populations with high genetic variability, but if the conditions are right (i.e. the scale of competition is sufficiently local), conflict can occur even within highly related groups (Gonzalez-Pastor et al. 2003; Griffin et al. 2004). Explanations for cooperation must take into account the ever-present potential for invasion and take-over by selfish cheats – individuals that avoid contributing to population-level cooperation while still deriving benefits from the cooperation of others (Velicer 2003; Travisano and Velicer 2004; West et al. 2006).

Invoking this sort of group selection to account for microbial social phenomena is both fallacious and unnecessary. In contrast, focusing on the costs and benefits accruing to individuals (and other individuals with whom they interact), can offer more insightful, more powerful and ultimately more biologically plausible hypotheses. For instance, the presence of genetic variants within biofilms may not reflect the endpoints of an adaptive programme of division of labour but simply

evidence of selection at work within the biofilm (Cooper et al. 2005; but see also Brockhurst et al. 2006). The seemingly altruistic production of exploitable resources such as extracellular polysaccharide (biofilm ‘slime’) could in fact be driven by conflicts within the biofilm, as cells compete to occupy the surface zones of the biofilm (Xavier and Foster 2007). The ‘persister cell’ phenomenon, meanwhile, could have evolved as a bet-hedging strategy benefitting the inclusive fitness of an individual cell because quiescence raises its own individual chances of surviving catastrophes (Gardner et al. 2007). Even apoptosis can be explained from the perspective of the inclusive fitness of an individual cell (Ackermann et al. 2008; Gardner and Kümmerli 2008). Quorum sensing too, did not necessarily evolve as a universally beneficial system of communication, but rather could have arisen as an adaptation whereby individual cells could gauge the diffusive properties environment (Redfield 2002) or coerce other cells into cooperating (Keller and Surette 2006).

Bringing rigorous and up-to-date social evolution theory into microbiology is important (Foster 2005; Nesse and Stearns 2008). Microbes, with their large populations and rapid generation times, can respond quickly to selection and if we want to properly understand the course this evolution will take, we will need to understand not only the proximate mechanistic details of microbiological phenomena, but also the ultimate evolutionary forces that govern the emergence and persistence of these traits of interest (Tinbergen 1963). The distinction between individual-based explanations for cooperation and those that rely solely on population-level benefits is crucial: because of the selfish interest of individuals, bacterial populations will not always evolve in ways that optimise population survival.

1.3. Aims of this thesis

In the preceding sections I have argued for a closer union between social evolution and microbiology. In this thesis I work towards achieving this aim, by (a) demonstrating how social evolution theory can be advanced by testing it in the

context of microbial systems (chapters 2 and 3) and (b) applying social evolution theory to further our understanding of microbial virulence (chapters 4 and 5).

Specifically:

- Chapter 2 considers the theoretical problem of how and when microbial cooperation might be subject to frequency-dependence. The chapter comprises a combination of theory and laboratory experiments which test this theory, using as a model system the cooperative production of siderophores (iron scavenging molecules) by the bacterium *Pseudomonas aeruginosa*.
- Chapter 3 follows a similar format to chapter 2 but explores a different theoretical question – that of when and why cooperation is expected to be density dependent. Again, I test theoretical predictions using *in vitro* experiments with the *P. aeruginosa* siderophore model system.
- Chapter 4 asks whether the virulence of multi-strain infections can be influenced by social conflicts among co-infecting pathogens. I tackle this question empirically, concentrating on the public goods type cooperative trait of siderophore production and using experimental infection models of different host models with the well-known generalist pathogen *P. aeruginosa*.
- Chapter 5 extends the work of the previous chapter to a different model trait, quorum sensing, which is widely held to be a key factor in the virulence of many microbial pathogens. I test whether social conflict over this trait, previously demonstrated to occur *in vitro*, also applies *in vivo* in an infection context, and whether it influences the resulting virulence of an infection. In this chapter I also evaluate, through my own experiments and a survey of the existing literature, the general importance of quorum sensing to *P. aeruginosa* virulence.

2. Frequency dependence and cooperation: theory and a test with bacteria[§]

2.1. Summary

Hamilton's inclusive fitness theory provides a leading explanation for the problem of cooperation. A general result from inclusive fitness theory is that, except under restrictive conditions, cooperation should not be subject to frequency-dependent selection. However, several recent studies in microbial systems have demonstrated that the relative fitness of cheats, which do not cooperate, is greater when cheats are rarer. Here we demonstrate theoretically that such frequency-dependent selection can occur in microbes when there is: (a) sufficient population structuring, or (b) an association between the level of cooperation and total population growth. We test prediction (b) and its underlying assumption, using the pathogenic bacterium *Pseudomonas aeruginosa*, by competing strains that produce iron scavenging siderophore molecules (cooperators) with non-producers (cheats) at various ratios, under conditions that minimise population structuring. We found that both the relative fitness of cheats, and the productivity of the mixed culture were significantly negatively related to initial cheat frequency. Furthermore, when the period of population growth was experimentally shortened, the strength of frequency dependence was reduced. More generally, we argue that frequency-dependent selection on cooperative traits may be more common in microbes than in metazoans because strong selection, structuring and cooperation-dependent growth will be more common in microbial populations.

2.2. Introduction

Explaining cooperation is one of the greatest challenges for evolutionary biology (Maynard Smith and Szathmàry 1995; Hamilton 1996). The problem is: why should an individual perform a cooperative behaviour that appears costly to perform, but benefits other individuals (Hamilton 1964)? Following a wealth of theoretical work

[§] Published as Ross-Gillespie, A., Gardner, A., West, S.A. and A. S. Griffin. 2007. Frequency dependence and cooperation: theory and a test with bacteria. *American Naturalist* 170:331-342. See appendix A2.

in this area in recent decades, we now have an excellent general understanding of the different ways in which cooperation can be favoured (reviewed by Frank 2003; Sachs et al. 2004; Lehmann and Keller 2006; West et al. 2006, 2007). However, much of this vast body of theory has been developed to elucidate general principles, and is less useful for stimulating empirical tests of theory (Leimar and Hammerstein 2006). Consequently, a major task for social evolution research is to take this general theory and develop it such that it can be tested empirically, with specific groups of organisms.

Microbes offer a number of advantages for testing social evolution theory (reviewed by Crespi 2001; West et al. 2006). In particular, they perform a number of cooperative behaviours, where it is possible to alter the costs and benefits of cooperation experimentally, and then follow the fitness consequences (West et al. 2006). Recently, there has been interest in how the relative fitness of cooperators, and cheats who do not cooperate (or cooperate less), depends upon the relative proportion of cooperators in the population (Velicer et al. 2000; Dugatkin et al. 2003; Dugatkin et al. 2005; Harrison et al. 2006; MacLean and Gudelj 2006). However, these experimental examinations of frequency dependence contrast with most theory in this area, where frequency dependence is not predicted. Indeed, a major result from Hamilton's (1964) original inclusive fitness formulation, showing how cooperation can be favoured between relatives, was that it did not vary with gene frequency at all. Instead, because increased cooperation also represents an increased opportunity for exploitation, the fitness advantage of cooperation remains constant despite changes in the frequency of cooperators. Hamilton was so pleased by this result that he later described it as a "gift from God" (Hamilton 1988). Subsequent work has shown that frequency dependence can enter into kin selection theory in cases featuring strong selection (genes with large fitness effects) or non-additivity (e.g. Charlesworth 1978; Michod 1982; Toro et al. 1982; Queller 1984; Frank 1998; Day and Taylor 1998; Rousset 2004).

Our first aim in this paper is to develop theory that predicts specifically when and why frequency dependent selection on cooperation should be observed in microbes.

The phenomenon is illustrated by considering the extracellular secretion of substances which can be utilised by neighbouring cells. The production of such ‘public goods’ requires explanation because they are costly to the individual to produce, but provide a benefit to the local group (West et al. 2006). In this situation, we might, at first glance, expect that selection on cells that contribute less than their fair share to the public good resource pool (cheats) should be frequency dependent. That is, cheats should do better when they are rare, because there will be more public goods / cooperators for them to exploit. However, cooperators also gain a benefit from being in a more cooperative population. In the simplest scenario, these two effects exactly cancel and so selection on cheats and cooperators does not depend on their frequency (see model section). We will examine the extent to which frequency dependence does arise when the following biological complexities are taken into consideration: population structure, effects of cooperation on population growth, and large (as opposed to small) fitness consequences associated with variation in the level of cooperation (strong selection).

Our model predicts that, given strong selection, cheats will be subject to negative frequency-dependent selection (i.e. their relative fitness will be lower when common) when (a) the population is structured, and / or (b) a higher frequency of cooperators leads to greater population growth. We tested prediction (b), and the underlying assumption on the relationship between growth and cooperator frequency, by investigating the production of a public good, iron-scavenging siderophore molecules, in the bacterial pathogen *Pseudomonas aeruginosa* (West and Buckling 2003; Griffin et al. 2004). Iron is a major limiting factor for bacterial growth, because most iron in the environment is in the insoluble Fe(III) form and is actively withheld by hosts (Guerinot 1994; Ratledge and Dover 2000). Siderophores scavenge insoluble and host-bound iron, making it available for bacterial metabolism. To determine whether frequency dependence is a general characteristic of *P. aeruginosa* siderophore production, and not just a pleiotropic effect associated with a specific cooperator-cheat pair, we examined three independently-derived cooperator-cheat strain pairs. These involved mutants produced by UV mutagenesis, single gene deletion, and spontaneous natural mutation respectively.

2.3. Models and Analyses

We will explore some ways in which frequency-dependent selection can arise in kin selection models for the production of public goods dilemmas in microbes. In particular, we examine the role of (1) population structure (including mixing rates and the scale over which social interaction occurs), and (2) the dependence of population carrying capacity and growth on the level of public goods contributions. In both cases, we allow for strong selection, which is crucial for frequency dependence in the context of social behaviours, and is an important characteristic of many microbial cooperative traits (see discussion). To begin, we demonstrate that frequency dependence does not emerge under the simplest possible scenario with weak selection.

Weak selection and frequency independence

In a broad class of models, weak selection leads to frequency independence (Rousset 2004, p80; 2006). To see why, consider a large population facing a public goods dilemma, such as, for example, siderophore production. An individual's fitness is a function of: (1) its level of public goods contribution, s ; and (2) the average public goods production across the whole population, \bar{s} . Without making further assumptions, we can express fitness as $w(s, \bar{s})$; this can be done for structured and unstructured populations. Further, we assume that cooperative individuals contribute an amount s_C , and that a proportion p of the individuals in the population are defectors (cheats) who contribute a smaller amount, $s_D = s_C - \delta$; hence, $\bar{s} = s_C - p\delta$. Thus, the fitness of a cooperator is

$$w_C = w(s_C, s_C - p\delta), \quad (1)$$

and the fitness of a cheat is

$$w_D = w(s_C - \delta, s_C - p\delta). \quad (2)$$

The relative fitness of cheats is given by the ratio of cheat and cooperator fitness and, using a Taylor expansion, can be expressed as:

$$v_D = \frac{w_D}{w_C} = 1 - \left. \frac{\partial w}{\partial \delta} \right|_{\delta=0} \delta / \hat{w} + O(\delta^2), \quad (3)$$

where $\hat{w} = w(s_C, s_C)$ is the fitness of cooperators in the absence of cheats. This reveals that, to leading order in δ , the relative fitness of cheats is not a function of their frequency p in the population (the partial derivative appearing in equation (3) is not a function of p , because it is evaluated in the neutral population where $\delta = 0$, so that cooperators and defectors are indistinguishable and the frequency of the latter is irrelevant). Hence, if selection is weak (δ sufficiently small for higher order terms in δ to be negligible), it is also frequency independent. For stronger selection, the trailing term of order δ^2 becomes more important, and if this is a function of the frequency of cheats then there is the possibility of frequency-dependent selection. Note that although our analyses focus on whether the fitness of cheats is frequency dependence, the fact that we are examining relative fitness means that they also demonstrate the nature of selection on cooperators – if the relative fitness of cheats decreases as they become more common, then the relative fitness of cooperators must be increasing as they become rarer.

Structured populations

One way in which strong selection can give rise to frequency-dependent selection is if populations are structured so that cooperators and cheats do not share the same social environment. This could occur in populations with localised social interaction due to limited mixing of public goods or cells. A consequence of this is that more cooperative individuals would have greater access to public goods, either because they enjoy preferential access to the public goods that they have produced, or because they use the public goods produced by their clonal relatives (who are also cooperators). We examine this in general terms, describing fitness as a product of

two components that capture: (1) the cost of producing public goods; and (2) the benefit of receiving public goods. In particular, we write:

$$w(s, \hat{s}) = g(s)h(\hat{s}), \quad (4)$$

where: w is fitness; g is a growth component that decreases with one's individual allocation to public goods production, s ; and h is a growth component that increases with one's access to public goods, \hat{s} (for related microbial models see Brown 1999; Smith 2001; West et al. 2002; Dugatkin et al. 2003; West and Buckling 2003). In mathematical terms, we have: $g > 0$, $\partial g / \partial s = g' < 0$, $h > 0$ and $\partial h / \partial \hat{s} = h' > 0$. We make the further assumption of linear or diminishing returns to fecundity as access to public goods in the social environment is increased, giving $\partial^2 h / \partial \hat{s}^2 \leq 0$. Due to population structure, the level of public goods available in the social environment is correlated with the individual's own allocation to public goods, according to the expression $\hat{s} = rs + (1 - r)\bar{s}$; i.e. the level of public goods available lies between one's own investment (s) and the population average investment (\bar{s}), according to the relatedness between social partners (r). The variable r encapsulates the effects of both bacterial dispersal and the diffusion of public goods through the population. It therefore allows us to move between the extremes of a completely mixed population or global diffusion of public goods ($r=0$), and a completely structured population, where all neighbours are the same genotype or public goods disperse over such small distances that they are only utilised by the individual that produced them ($r=1$; cheats only interact with cheats, and cooperators only interact with cooperators).

We compare the fecundity of a strain that allocates nothing to public goods ($s = 0$, defection) to that of a strain that allocates a standard unit ($s = 1$, cooperation).

Respectively, these are:

$$w_D = g(0)h((1 - r)(1 - p)) = g_D h_D, \text{ and} \quad (5)$$

$$w_C = g(1)h(r + (1 - r)(1 - p)) = g_C h_C, \quad (6)$$

where p is the population frequency of cheats, and hence $\bar{s} = 1 - p$ is the population average allocation to public goods, being the proportion of cooperators.

The relative fitness of cheats can be written as:

$$v = \frac{w_D}{w_C} = \frac{g(0)h((1-r)(1-p))}{g(1)h(r+(1-r)(1-p))} = \frac{g_D h_D}{g_C h_C}. \quad (7)$$

We now determine whether and how the relative fitness of cheats (v) varies with their frequency in the global population (p):

$$\frac{dv}{dp} = \frac{g_D}{g_C} (1-r) \frac{h_D h'_C - h_C h'_D}{h_C^2}. \quad (8)$$

This reveals the following: (1) In the absence of population structure ($r = 0$) there is no frequency dependence ($dv/dp = 0$), because cooperators and cheats share the same social environment and hence $h_C = h_D$ and $h'_C = h'_D$; (2) In fully-structured populations ($r = 1$) there is no frequency dependence ($dv/dp = 0$), because an individual's social environment is dependent only on its own cooperation strategy and not on the global frequency of cooperators; (3) In a population with intermediate structuring ($0 < r < 1$) there is negative frequency dependence ($dv/dp < 0$), because cooperators enjoy more public goods in their social environment than do cheats and hence $h_C > h_D$ and $h'_C \leq h'_D$.

The impact of population structure (r) on cheat relative fitness (v) is examined using the same approach. The derivative

$$\frac{dv}{dr} = -\frac{g_D}{g_C} \frac{p h_D h'_C + (1-p) h_C h'_D}{h_C^2} \quad (9)$$

is a negative quantity, and hence increasing population structure (higher r) reduces the relative fitness (v) of cheats. This is because cheats will have a higher fitness in more mixed populations, where they are better able to exploit the cooperators. This

could be tested empirically by examining the strength of frequency dependence across populations that are structured to different degrees – e.g. using shaken versus unshaken liquid culture media, or semisolid agar culture media of varying viscosities. Numerical illustrations of the model are given in Figure 2.1.

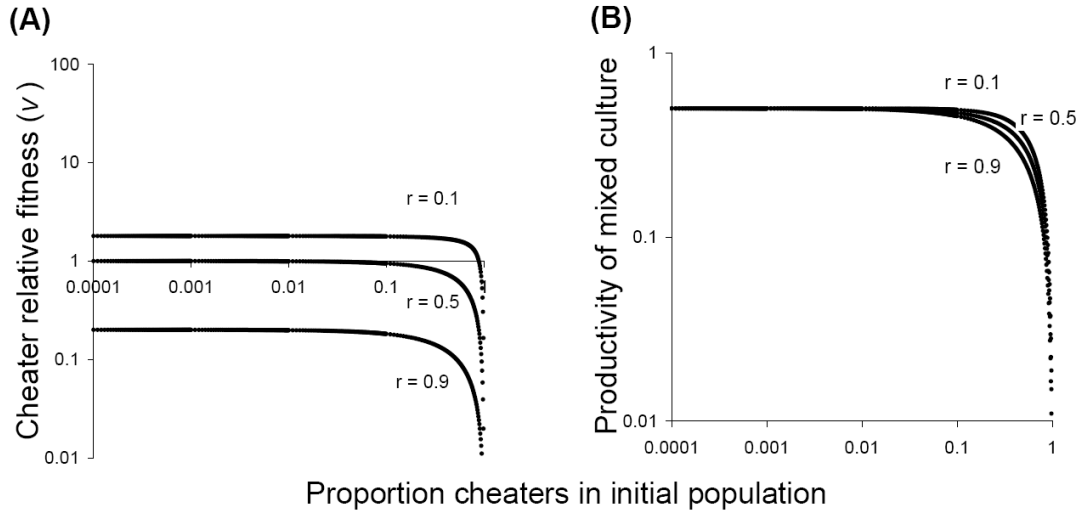


Figure 2.1. Structured-population model, with fitness given by the product of $g[s] = 1 - s/2$ and $h[\hat{s}] = \hat{s}$ where s is an individual's own contribution to public goods, and \hat{s} is the amount of public goods in the individual's social environment. (A) The relative fitness of cheats (v) decreases with their frequency (p) and with the degree of population structure (r). (B) The total productivity of the population decreases with the frequency of cheats (p) and with degree of population structure (r).

Population growth as a function of cooperation frequency

Another way in which strong selection can give rise to frequency dependence is when the growth of a population depends on its genetic composition. If the growth of a bacterial population is negligible when cheating is prevalent, we expect little change in population size and genetic composition, due to cheats having a limited opportunity to exploit cooperators. Conversely, if more cooperative bacterial colonies achieve a higher carrying capacity and hence more growth, differences in growth rates of cooperators and cheats could lead to more pronounced changes in gene frequencies. Describing fitness in terms of absolute increase over the growth period, the relative fitness of faster growers increases with the number of rounds of

division. Put simply, more growth means a greater chance for cheats to exploit cooperators.

For simplicity, consider a well-mixed population of bacteria growing exponentially over a time period that depends on the initial proportion of cooperators. Defining a basic time unit such that the instantaneous rate of growth is 1 for cooperators, then the growth rate of cheats can be represented as $1+b$. After t time units, the numbers of cooperators and cheats are given by:

$$n_{C,t} = n_{C,0}e^t, \quad (10)$$

$$n_{D,t} = n_{D,0}e^{(1+b)t}, \quad (11)$$

respectively, where $n_{C,0}$ and $n_{D,0}$ are, respectively, the numbers of cooperators and cheats at time $t = 0$. If growth ceases at time $t = T[p_0]$, which decreases with the initial proportion of cheats (p_0 ; i.e. $dT/dp_0 < 0$), then the frequency of cheats following growth is given by:

$$p_T = \frac{n_{D,t}}{n_{D,t} + n_{C,t}} = \frac{p_0 e^{bT}}{p_0 e^{bT} + 1 - p_0}. \quad (12)$$

Hence, the relative fitness of cheats versus cooperators is:

$$v = e^{bT}. \quad (13)$$

It is easy to show that relative fitness of cheats is frequency dependent, and indeed that it is a decreasing function of cheat frequency:

$$\frac{dv}{dp_0} = \frac{\partial v}{\partial T} \frac{dT}{dp_0} = be^{bT} \frac{dT}{dp_0} \quad (14)$$

is negative because $dT/dp_0 < 0$. Note that frequency independence is recovered in the limit of weak selection (small b). Here, we can write $T = \hat{T} + O(b)$ where \hat{T} is a constant with respect to the frequency of cheats, p . Hence, from a Taylor expansion of (13), relative fitness is given by $v = 1 + \hat{T}b + O(b^2)$, i.e. to a first order approximation it is independent of the frequency of cheats.

This model of exponential growth, with an abrupt halt at a time depending on the population level of cooperation, is unrealistic and pursued only for the sake of analytic tractability. We now consider a more realistic model describing logistic growth and competition for resources, in which a population of cooperators can maintain a higher cell density than can a population of cheats. In particular, we continue to assume an intrinsic growth rate of 1 and $1+b$ for cooperators and cheats respectively, but also include a density-dependence term in the dynamical equations describing growth:

$$\frac{dn_C}{dt} = n_{C,t}(1 - E_t), \quad (15)$$

$$\frac{dn_D}{dt} = (1 + b)n_{D,t}(1 - E_t), \quad (16)$$

where: the numbers of cooperators and cheats ($n_{C,t}$ and $n_{D,t}$) are expressed as proportions of the total population size that can be maintained at equilibrium (carrying capacity) if all cells cooperate; and $E_t = n_{C,t} + (1 + a)n_{D,t}$ is the ‘effective’ population size, in terms of the strain on resources that slows population growth, where each cheating individual incurs a strain that is equivalent to that of $1+a$ cooperators. This reflects the benefit of cooperation for the population: for $a > 0$, defectors inflate the effective size of the population, so that when they are common the actual number of cells that can be sustained at stationary phase is reduced. This model contains nonlinearities which prevent an analytical treatment, though numerical solutions are possible (Figure 2.2), and these recover the same qualitative results derived above for the simpler exponential growth model.

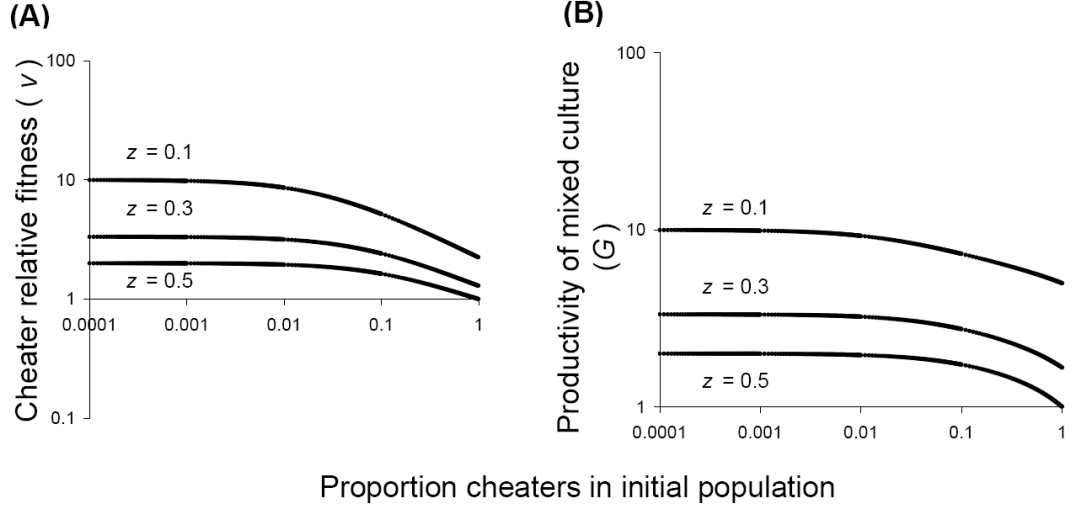


Figure 2.2. Logistic growth model, with more cooperative populations experiencing higher carrying capacity, for a range of initial population sizes ($z_0 = n_{C,0} + n_{D,t}$ as a proportion of carrying capacity for a fully-cooperative population) and initial proportion of cheats ($p_0 = n_{D,0}/z_0$). We assume that cheats grow at twice the rate of cooperators, and that they exert twice as great a competitive strain on resources ($a = b = 1$). (A) The relative fitness of cheats (v) decreases as initial population size (z_0) and initial proportion of cheats (p_0) increases, because both result in reduced population growth, and hence less pronounced fitness differences. (B) The total productivity of the population (G) decreases as initial population size (z_0) and initial proportion of cheats (p_0) increases, because both result in reduced population growth.

2.4. Methods

We experimentally tested our prediction that cheat fitness will be negatively correlated with the frequency of cheats when population growth increases with cooperator frequency. We minimised the possible effects of structured populations leading to frequency dependence by carrying out growth in a shaken liquid media.

Description of strains

Three cooperator-cheat pairs (“A” to “C”; see appendix A1), comprising strains of *Pseudomonas aeruginosa* that differed with respect to production of the primary siderophore, pyoverdine (pvd), were used in this experiment. For convenience, we will further denote individual strains as either “+” (cooperator, did produce pvd) or “-”

” (cheat, defective for pvd), giving A+, A-, B+, B-, C+, and C-. Specifically, the strains used were as follows: A+: PA01 (strain ATCC15692), a pvd-producing wildtype; A-: PAO6609 (Hohnadel et al. 1986), a pvd-negative mutant derived by UV-mutagenesis from methionine auxotroph PAO6409 (Rella et al. 1985), which in turn was generated by transposon mutagenesis from PA01; B+: PA01 (strain ATCC15692), pvd-producing wildtype, as for A+ above; B-: an unmarked deletion mutant derived from PA01, defective for the pyoverdine synthetase gene *pvdD* (Ghysels et al. 2004); C+: a wildtype, pvd-producing clone cultured from UCBPP-PA14, a clinical isolate known to also be pathogenic in plants and animals (Rahme et al. 1995). C-: a pvd-negative mutant coevolved with C+ in the laboratory under iron-limiting conditions, which should favour the spread of spontaneously arising mutants that somehow avoid the cost of producing siderophores and instead take up those produced by neighbouring bacteria. Both C+ and C- were isolated from the same overnight culture, which at that point had been passaged through 19 (daily) serial transfers, where each transfer entailed the inoculation of 60 µl of overnight culture (incubated at 37°C and shaken at 200 r.p.m.) into 30ml glass universal vials containing 6 ml fresh medium (CAA; 5g casamino acids, 1.18g K₂HPO₄·3H₂O, 0.25g MgSO₄·7H₂O per litre). Like A- and B- which are known to be pvd-synthesis defective, mature C- colonies also have a pale white appearance, distinguishing them from both C+ and their parent strain, which appear conspicuously green owing to the presence of pyoverdine.

Experimental design

For each strain pair, + and - cultures were initiated from freezer stock and incubated overnight in an orbital shaker (37°C, 200 r.p.m.). For this first stage, strains were grown in 6 ml standard Kings B solution in 30 ml glass universal vials. After 24 hours, the cultures were retrieved and vortexed for 45 seconds. For pair A only, pilot data predicted substantial cell density differences between the A+ and A- solutions after 24 hours growth in KB, so to equalise cell densities in the + and - cultures, a volume of A+ solution (3.64 ml) was drawn off and replaced with buffer solution (M9).

We then prepared, by appropriate dilution, “treatment” cultures in which – (cheats) were mixed with + (cooperators) at cell density ratios of approximately 1:1000, 1:100, 1:10, 1:1, and 100:1 respectively. These solutions were supplemented with buffer (M9) such that each would contain cells at approximately the same density ($\sim 10^6$ cells/60 μ l). For each of these five treatments, we inoculated 6 replicate “competition” vials with 60 μ l of the prepared mixed cultures: 30 ml universal glass vials containing 6 ml CAA medium as described previously, supplemented with 100 mg.ml⁻¹ human apo-transferrin (an iron chelator) and 20 mM NaHCO₃, necessary for effective chelator activity (Meyer et al. 1996). The 30 competition vials were then placed, in random order, in an orbital shaker for overnight incubation (37°C, 200 r.p.m.).

To assess the initial ratios of cooperators to cheats in the inocula, multiple samples were taken, grown on KB-agar plates, and colony-forming units (CFUs) were counted and categorized as either + or - on the basis of colour and morphology.

Similarly, each of the 30 competition cultures, after 24 hours of incubation at 37°C, was diluted, spread on to KB-agar plates, incubated again overnight for counting. In this way, we obtained data on the absolute density and relative proportions of + and – CFUs both before and after the competition period. Labelling, spreading and counting of plates, at each stage, were fully randomized to minimize order effects.

For pairs A and B, a single round of competition was conducted. In the case of pair C, two rounds were conducted, in each of which a different treatment failed to yield useful data. The results were of both rounds were thus pooled for combined analysis.

We also carried out a separate experiment to test whether reducing the period of population growth reduced the extent of frequency dependence. Using strain pair A, we simultaneously inoculated two parallel sets of three treatments, with cheat:cooperator ratios of approximately 1:1000, 1:10 and 100:1 respectively. Our protocol was identical to that described above, except that, whereas one set was

cultured for a full 24 hours before sampling, the second set was sampled after just 6 hours of incubation.

Statistical analyses

We performed two types of analyses on data from each of the three strain pairs. Firstly, we calculated relative cheat fitness (v), by comparing the frequency of cheats at the beginning and end of the experiment. Specifically, v is given by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_I is the initial proportion of cheats in the population, x_2 is their final proportion. The value of v therefore signifies whether cheats increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We assessed the nature and strength of the relationship between v and x_I , using standard General Linear Models (GLMs) with x_I as the explanatory variable. In each case, v and x_I were first log-transformed or – in the case of pairs 1 and 2 – converted to the form $\log(v+1)$ to normalise the distribution of the residuals, in accordance with the assumptions of parametric analyses. In the case of pair C, “round” was included as a blocking factor in the maximal model, but was non-significant, and so was excluded from the final model. Secondly, we quantified productivity (G) as the final cell density attained by the mixed culture at the end of the competition period. Again, this was analysed with respect to initial cheat frequency, x_I , using a standard GLM. Here again, G was first log-transformed to normalise the distribution of the residuals. All analyses were performed with MINITAB 14.1 (www.minitab.com).

2.5. Results

In all three cheat-cooperator pairs, the relative fitness of cheats was significantly negatively correlated with initial cheat frequency (Figure 2.3A). In all cases, the relationship was best described by a power function (pair A: $v = 4.200x_I^{-0.442}$, GLM: $F_{1,28} = 92.17$, $p < 0.001$; pair B: $v = 1.856x_I^{-0.123}$, GLM: $F_{1,26} = 11.27$, $p = 0.0024$; pair C: $v = 2.270x_I^{-0.176}$, GLM: $F_{1,45} = 37.24$, $p < 0.001$). At low frequencies, cheats showed higher fitness than cooperators; while at high frequencies, cheat fitness was

comparable to – or, in the case of pair A – significantly lower than – that of cooperators.

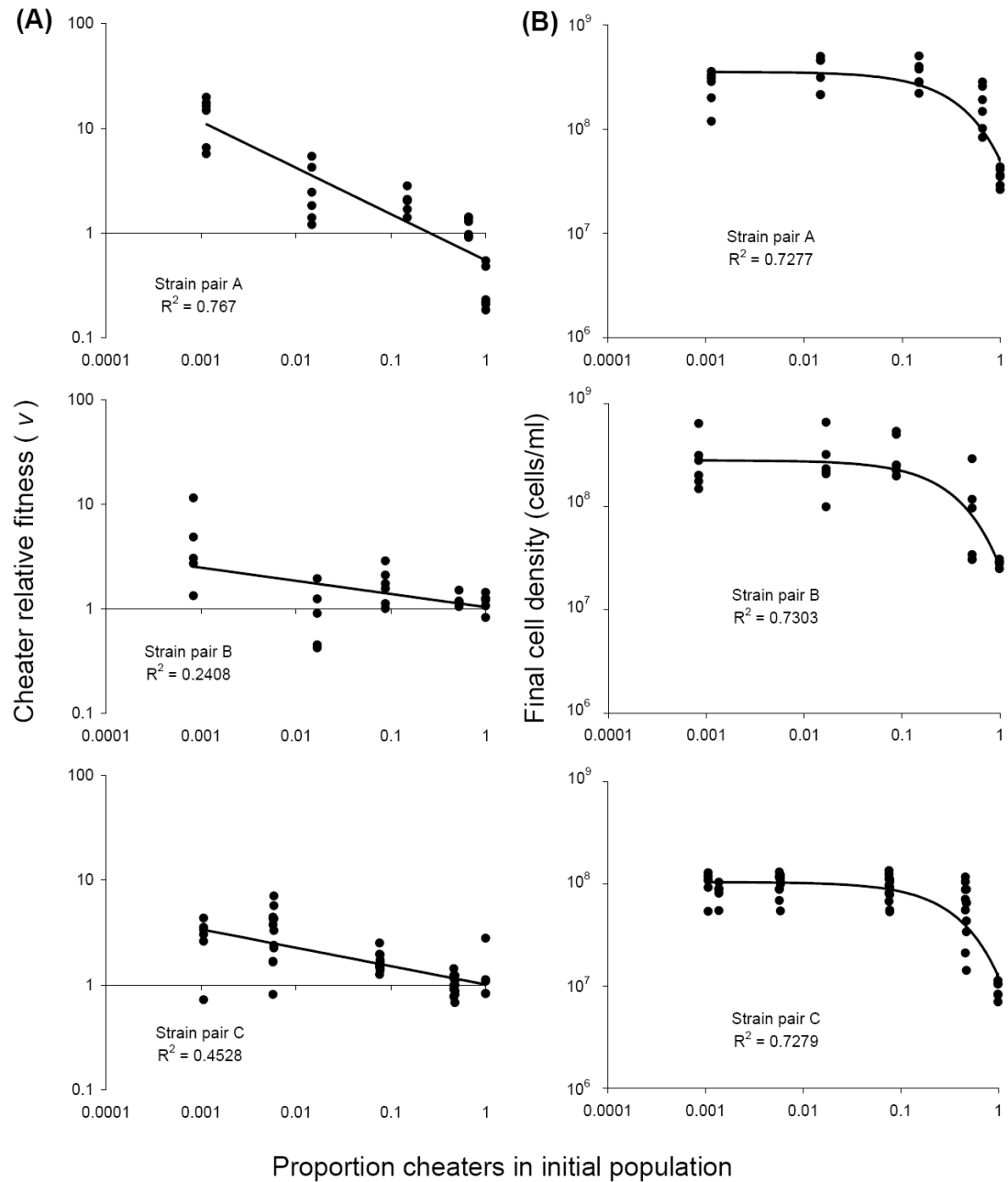


Figure 2.3. (A) Relative cheat fitness and (B) mixed-culture productivity as a function of initial cheat frequency, for three independent pairs of *Pseudomonas aeruginosa* strains. Here, relative fitness is the proportional increase in frequency of cheats relative to cooperators, and productivity is the final cell density of the mixed culture after a 24 hour competition period. Fitted lines reflect (A) power and (B) exponential regression curves, estimated by least-squares.

For all three strain pairs, productivity of the mixed culture was significantly negatively correlated with initial cheat frequency (Figure 2.3B). In all cases, the relationship was best described by an exponential function (pair A: $G = 3.56 \times 10^8 e^{-1.97x_I}$, GLM: $F_{1,28} = 74.83$, $p < 0.001$; pair B: $G = 2.82 \times 10^8 e^{-2.38x_I}$, GLM: $F_{1,26} = 75.82$, $p < 0.001$, $r^2 = 0.70$; pair C: $G = 1.04 \times 10^9 e^{-2.13x_I}$, GLM: $F_{1,51} = 139.11$, $p < 0.001$). In cultures initiated with a high frequency of cheats (i.e. the 100:1 treatment), productivity was around an order of magnitude lower than in cultures where cheats were scarce (i.e. 1:1000 treatment).

The strength of frequency dependence was reduced when populations were allowed to grow for shorter periods of time (Figure 2.4). In our further experiment with strain pair A, the population growth was significantly lower in the 6hr old cultures than in the 24hr old cultures (Figure 2.4B; 24-hour cultures: $G = 1.01 \times 10^8 e^{-2.779x_I}$, 6-hour cultures: $G = 1.68 \times 10^6 e^{-3.824x_I}$; GLM: final density*time $F_{1,29} = 119.76$, $p < 0.001$). The strength of frequency dependence with respect to cheat relative fitness was also reduced over the shorter time period (Figure 2.4A; 24-hour cultures: $v = 1.303x_I^{-0.241}$, 6-hour cultures: $v = 1.117x_I^{-0.036}$; GLM: fitness*time $F_{1,27} = 13.06$, $p < 0.001$).

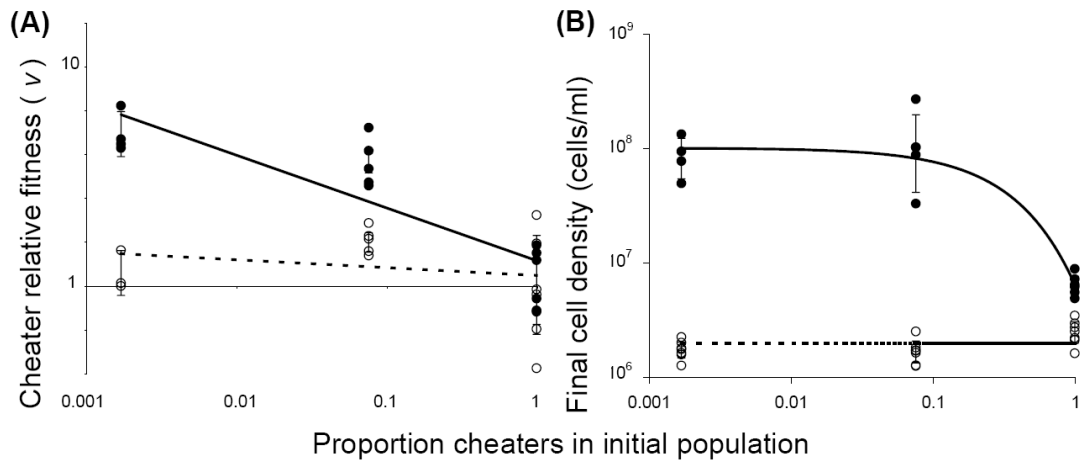


Figure 2.4. (A) Relative cheat fitness and (B) mixed-culture productivity as functions of initial cheat frequency, in 6 hour cultures (open circles and dotted regression line) versus 24 hour cultures (filled circles and solid regression line). Here, relative fitness is the proportional increase in frequency of cheats relative to cooperators, and productivity is the final cell density of the mixed culture at the end of the competition period. Fitted lines reflect (A) power and (B) exponential regression curves, estimated by least-squares. Error bars reflect 95% confidence intervals around the means.

We carried out a number of additional analyses to test the robustness of our results. First, we considered an alternative measure of productivity: the fold increase in total cell density over the competition period. These analyses produced results qualitatively concordant with those presented here. Second, we considered an alternative approach for all fitness vs. proportion analyses: instead of regressing x_I against v (an expression which itself contains x_I), we simply regressed the initial odds ratio, $x_1/(1-x_1)$ against the final odds ratio, $x_2/(1-x_2)$. By this approach, fitted regression lines with slopes significantly less than 1 indicate negative frequency dependence. In the first set of experiments, we found this result for all three strain pairs (pair A: slope 0.73 ± 0.02 , $T_s = 12.30$, d.f. = 28, $p < 0.001$; pair B: slope 0.93 ± 0.03 , $T_s = 2.32$, d.f. = 26, $p = 0.029$; pair C: slope 0.84 ± 0.04 , $T_s = 4.33$, d.f. = 48, $p < 0.001$). In the experiment considering different competition periods, only the 24 hour cultures showed negative frequency dependence (24 hr cultures: slope 0.84 ± 0.02 , $T_s = 7.68$, d.f. = 16, $p < 0.001$; 6 hour cultures: slope 0.97 ± 0.03 , $T_s = 1.29$, d.f. = 14, $p = 0.218$). Third, we repeated all analyses using the ratio of cheats to cooperators (x_R) as the explanatory variable, rather than the proportion of cheats in the mixed population (x_I), where $x_R = x_1/(1-x_1)$. For all three strain pairs, results obtained in regressions with x_R were qualitatively analogous to those presented here.

2.6. Discussion

In this study, we have determined under what conditions the production of public goods in microbes will be subject to frequency-dependent selection. Specifically, we have shown that if there is strong selection, two sets of conditions can lead to frequency-dependent fitness: (a) when cooperators acquire a more than a random benefit of public good production – e.g. if cooperators tend to be clustered together (population structuring) or if individual cells have preferential access to the public goods that they produce (Figure 2.1); or (b) when increased levels of cooperation lead to greater growth, a higher carrying capacity, and hence more generations over which cheats can exploit cooperators (Figure 2.2). In both cases, the relative fitness of cooperators or cheats is greater when they are less common. We then explicitly tested the second of these possibilities (by controlling for the first, population

structuring), using the production of iron scavenging siderophore molecules in *P. aeruginosa* as a model trait. As predicted, we found a negative relationship between the relative fitness of cheats and the proportion of cheats in the population (Figure 2.3A). Furthermore, we found support for the underlying assumption leading to this prediction - a higher proportion of cooperators led to greater growth (Figure 2.3B), and a reduction in the period of growth led to a decrease in the extent of frequency dependence (Figure 2.4).

Situations favouring frequency-dependent selection

We have identified two ways in which kin selection models of public goods dilemmas can generate frequency-dependent selection, given strong selection. Firstly, population structure, incorporating both limited mixing of cells and limited diffusion of public goods molecules (local social interaction) can result in the selective advantage of cheating decreasing as cheats become more common. When populations are structured, the level of public goods available to an individual will depend upon the production of public goods by: (a) that individual and its clonal relatives; (b) the average level of public goods production in the population. Increasing population structure increases the importance of the former and decreases the importance of the latter. A higher frequency of cheats will lead to a lower average level of public goods production (b). The consequences of this will be greater for cheats, because they receive fewer public goods through their own production and that of their relatives (a). This leads to the relative fitness of cheats decreasing with cheat frequency. Secondly, frequency dependence may also arise when total population growth increases with the level of cooperation in the population. If more cooperative populations achieve a higher carrying capacity, then more rounds of growth and division are possible before stationary phase is reached. The selective benefit of cheating multiplies with each round of division, so that the relative fitness of a cheat measured over the period of population expansion increases with the (initial) frequency of cooperators in the population (Figure 2.2A).

How does our work relate to previous theory? We have provided some specific cases for the general conclusion that the generation of frequency-dependent selection relies on strong selection (Charlesworth 1978; Michod 1982; Toro et al. 1982; Queller 1984; Day and Taylor 1998; Nowak and May 1992; Rousset 2004; Jansen and Van Baalen 2006). The aim of this previous work was to elucidate general points, and so scenarios were modelled which do not facilitate simple application to specific systems such as public goods production (e.g. the two-player games; Queller 1984). An important exception to this was provided by Dugatkin et al. 2003), who explicitly considered frequency-dependent selection in microbes. However, Dugatkin et al. (2003) did not explain how frequency dependence should occur in the first place – their aim was to address the consequences of frequency-dependent selection, and so they simply assumed that it occurred. This is analogous to taking equation 7 or 16 as a starting point, rather than determining how they should arise. More generally, frequency dependence does not usually arise from most of social evolution theory, because selection is usually assumed to be weak, to make analyses more tractable (Hamilton 1964; Taylor and Frank 1996; Frank 1998; Rousset 2004). Frequency dependence can also arise in models for the evolution of cooperation that do not rely on kin selection (e.g. reciprocity; Axelrod and Hamilton 1981).

Frequency-dependent selection in microbes

Frequency-dependent selection can, under certain conditions, prevent cheat takeover and facilitate the co-existence of multiple phenotypes (Aviles 2002). However, in two of the three strain pairs examined in this study, cheats were at least as fit as cooperators, even when common (Figure 2.3A). With such a payoff structure, we may anticipate that, in time, cheats would go to fixation in populations where there is little competition between groups. Consistent with this idea, siderophore-defective strains are routinely isolated from the lungs of late-stage cystic fibrosis patients, where they typically grow as monoclonal populations (Lee et al. 2005; Salunkhe et al. 2005; Smith et al. 2006). On the other hand, in an environment where competition occurs between subpopulations, and these subpopulations contain different proportions of cheats, those groups with more cooperators may outcompete cheat-

dominated groups (Griffin et al. 2004). Thus, cooperation may also be maintained by frequency-dependent selection at the level of the group.

We suggest that negative frequency dependence should be common in microbes, because: (a) both population structuring and cooperation dependent growth rates are likely to be important in many situations; (b) mutations can occur which lead to large differences in the level of cooperation (strong selection; see below). Accordingly, negative frequency-dependent selection has now been shown for social cheats in at least nine studies on seven microbial species (Velicer et al. 2000; Vulic and Kolter 2001; Fiegna and Velicer 2003; Rainey and Rainey 2003; Turner and Chao 2003; Dugatkin et al. 2005; Harrison et al. 2006; MacLean and Gudelj 2006; this study). However, while we highlight these studies as support for our prediction, we also point out that most did not explicitly test the underlying assumptions of why frequency dependence occurs. Ultimately, a range of complementary approaches is most useful. At one extreme will be studies designed to reveal those specific mechanisms responsible for frequency dependence. For example, our aim in the present study was to test one particular mechanism that can lead to frequency dependence – cooperation-dependent population growth. In order to do this we (a) carried out our experiment in shaken vials, in order to remove the other possible mechanism that can lead to frequency-dependent selection – population structure; and (b) also tested the underlying assumption that a higher proportion of cooperators allows the population to grow to a larger size (Figure 2.3B). At the other extreme are studies in more natural conditions that examine the net consequences of both mechanisms for generating frequency dependence (e.g. Harrison et al. 2006). An important task for the future is to determine the relative importance in nature of the two different mechanisms that we have shown can lead to frequency dependence.

We observed a decrease in the extent of frequency dependence when the period of time available for growth was shortened (Figure 2.4). This supported the assumption of our model that the relative fitness of cheats increases over time as the population goes through more rounds of growth and division. It is possible that other factors could also cause the relative fitness of cheats to vary over time. For example, as the

population grows this could lead to a decrease in iron availability that reduces the relative fitness of cheats (Griffin et al. 2004), or a higher population density that allows cheats to better exploit cooperators, and hence increases the relative fitness of cheats (Greig and Travisano 2004; MacLean and Gudelj 2006). However, effects such as these cannot explain the frequency dependence evident in our data - in order for frequency dependence to be observed, one of the additional factors that we have suggested would still be required, such as populations with greater frequencies of cooperators growing to higher final densities.

More generally, our study illustrates that, although the same social evolution theory originally developed for metazoans can be applied to microbes, certain aspects of microbial biology mean that some differences may occur in the evolution of social traits. In particular, with typical social evolution study organisms, such as insects, birds and mammals, it is usually assumed that mutation will lead to minor variations in behaviour (weak selection), and so frequency dependence will be relatively unimportant (Sinervo and Lively 1996; Sinervo et al. 2006; Sinervo and Calsbeek 2006). In contrast, in the case of microbes, single or small numbers of mutations can have extremely large effects on social behaviours (strong selection; Velicer et al. 1998; 2000; Rainey and Rainey 2003; Velicer and Yu 2003; Foster *et al.* 2004; Griffin et al. 2004; Fiegna et al. 2006). For example, in *P. aeruginosa*, social behaviours such as siderophore production are routinely and completely lost from isolates obtained from lung infections (Lee et al. 2005; Salunkhe et al. 2005; Smith et al. 2006). Furthermore, the majority of microbial cooperative behaviours are controlled by quorum sensing regulatory networks (Keller and Surette 2006; Venturi 2006). Consequently, the disruption of these networks, even through the loss of a single gene, can have huge effects on the level of cooperation. A consequence of the potential for mutations of large effects (strong selection) in microbes, is that this allows frequency-dependent selection, through the mechanisms we have described in this study.

3. Density dependence and cooperation: theory and a test with bacteria^{**}

3.1. Summary

Whilst cooperative systems can persist in nature despite the potential for exploitation by non-cooperators, it is often seen that small changes in population demography can tip the balance of selective forces for or against cooperation. Here we consider the role of population density in the context of microbial cooperation. First, we account for conflicting results from recent studies by demonstrating theoretically that: (a) for public goods cooperation, higher densities are relatively unfavourable for cooperation; whereas; (b) for self-restraint type cooperation, higher densities can be either favourable or unfavourable for cooperation, depending on the details of the system. We then test our predictions concerning public goods cooperation using strains of the pathogenic bacterium *Pseudomonas aeruginosa* that either produce iron-scavenging siderophore molecules (cooperators) or do not (cheats). As predicted, we found that the relative fitness of cheats was greatest at higher population densities. Furthermore, as assumed by theory, we show that this occurs because cheats are better able to exploit the cooperative siderophore production of other cells when they are physically closer to them.

3.2. Introduction

Explaining the evolution and maintenance of cooperation is one of the most important and long-standing problems in evolutionary biology (Maynard Smith and Szathmàry 1995; Hamilton 1996). Whilst a profusion of theoretical work in the past five decades has led to a good theoretical overview of the different mechanisms by which cooperation can arise and persist, much of this theory remains to be tested (Sachs et al. 2004; Lehmann and Keller 2006; West et al. 2007b). One area that has attracted a lot of theoretical attention is how selection for cooperation can be affected

^{**} A version of this chapter has been submitted for publication: Ross-Gillespie, A., Gardner, A., Buckling, A., West, S.A. and A. S. Griffin. Density dependence and cooperation: theory and a test with bacteria.

by the ecology and demography of populations (see reviews by Frank 1998; Rousset 2004 and Lehmann and Keller 2006). Genetic composition, density, sub-structuring, and the degree of mixing between individuals can all affect the spread and stability of behavioural strategies within a population; but it can be difficult to predict, from such general theory, precisely how such effects would apply in the context of particular cooperative systems (Leimar and Hammerstein 2006; West et al. 2007b). Moreover, it has been difficult to test these ideas empirically, because of the inherent complexities of experimentally manipulating population structure in traditional model systems such as vertebrates or insects.

In this paper, we focus on the role of population density in the evolution of cooperation. We develop theory specific to the context of microbial cooperative systems and then test our predictions experimentally using a bacterial model system. It has recently been realised that microbes exhibit a striking range of cooperative behaviours, which offer excellent opportunities for testing social evolution theory (Crespi 2001; West *et al.* 2006). This is because mutants that do not cooperate (cheats) can be isolated from natural populations or artificially generated, the costs and benefits of cooperation can be easily manipulated experimentally, and the resultant fitness consequences monitored over many generations (Velicer 2003; West et al. 2006; Foster et al. 2007). Microbes are particularly useful for investigating the role of population structure in the evolution of cooperation, and recent experiments have provided clear support for the importance of factors such as substructuring, mixing and disturbance frequency (Griffin et al. 2004; Brockhurst et al 2007; Diggle et al. 2007b), and the relative frequency of cooperators vs. cheats in a population (Velicer et al. 2000; MacLean and Gudelj 2006; Diggle et al. 2007b; Ross-Gillespie et al. 2007;). With population density, however, the picture is less clear. While one recent study in yeast reported cheats having a higher relative fitness at higher population density (positive density dependence; Greig and Travisano 2004), another yeast study found that cheats had a higher relative fitness at lower cell densities (negative density dependence; MacLean and Gudelj 2006; MacLean 2008).

Our first objective in this paper is to develop explicit theory, tailored to microbial systems, that will make it clear when, why, and what sort of density dependence is expected. Specifically, we formalise why different patterns of density dependence are to be expected with different types of cooperative behaviour. In the first scenario, individuals cooperate by manufacturing products that can be utilised by their neighbours (i.e. public goods). This is a relatively common form of cooperation in microbes, with bacteria producing numerous factors that are released into the environment beyond the cell membrane (West *et al.* 2007a). In the context of a public goods dilemma, cheats prosper only insofar as they have access to the public goods produced by other individuals, and hence they are expected to perform relatively poorly at low population density and relatively better when the population density is higher (i.e. positive density dependence). In contrast, we suggest that different predictions can arise when the cooperative dilemma concerns the consumption of a finite public resource (the ‘tragedy of the commons’ scenario; (Hardin 1968), another situation that is commonplace in microbes (West *et al.* 2007a; MacLean 2008). In this context, cooperators are cells that show “self-restraint” and use the resource more efficiently, as opposed to quickly but relatively inefficiently. At low population density, resources will be less limiting, so there is no benefit to be had from more prudent use of resources, whereas at higher density, efficient resource use enhances the overall productivity of a social group and hence cooperation provides a greater benefit. In this case, cheats are expected to do better at lower densities (negative density dependence).

In the second part of the paper, we test our prediction that the production of a public good leads to the relative fitness of cheats, who do not produce it, being positively density dependent. We used as our model cooperative trait the production of siderophores in the bacterial pathogen *Pseudomonas aeruginosa* (West and Buckling 2003; Griffin *et al.* 2004). Siderophores scavenge iron from the environment, most of which is otherwise unavailable to bacteria since it is either actively withheld by hosts or is in the insoluble Fe(III) form (Guerinot 1994; Ratledge and Dover 2000). Siderophores are secreted extracellularly, diffuse freely and be taken up by any neighbouring cell. Previous work has shown that siderophore production is a

cooperative behaviour, vulnerable to exploitation by cheats (Griffin et al 2004; West et al. 2007c). As well as testing for positive density-dependence, we test the underlying assumption of our theoretical model: that cheat fitness is contingent on the physical proximity to cooperators and the siderophores that they produce. While our theoretical work examines both public goods and self-restraint, we test only the public goods model, because that is where the clearer *a priori* prediction can be made, and because this is part of our longer term project on the evolution of siderophore production.

3.3. Models and analyses

Relative fitness of cheats

Let the personal fitness w of an individual cell be a function of its cooperation strategy X and the average cooperation strategy Y of its social group, i.e. $w = W(X, Y)$. We define a ‘cooperator’ as investing more, and a ‘cheat’ as investing less, into cooperation. In particular, we assume that cheats invest an amount x into cooperation while cooperators invest $x + \delta x$, where $\delta x > 0$, and we denote the average strategy of the cheat’s social group as y and the cooperator’s social group as $y + \delta y$. Thus, the relative fitness of cheats can be expressed as:

$$v(x, y, \delta x, \delta y) = \frac{W(x, y)}{W(x + \delta x, y + \delta y)} = 1 - \left(\frac{\partial W}{\partial X} \delta x + \frac{\partial W}{\partial Y} \delta y \right) / W(x, y) + O(\delta^2), \quad (1)$$

where the partial derivatives are evaluated at $X = x$ and $Y = y$. Noting that $\delta y / \delta x = dY/dX + O(\delta)$, and denoting $dY/dX = R$, i.e. the ‘whole-group’ relatedness (this is the relatedness of an individual to the group, including itself – formally, the expected relatedness of two cells randomly drawn from the social group, with replacement; Taylor and Frank 1996; Pepper 2000; Rousset 2004), relative fitness can be expressed in the form:

$$v(x, y, \delta x, \delta y) = 1 - \left(\frac{\partial W}{\partial X} + \frac{\partial W}{\partial Y} R \right) \delta x / W(x, y) + O(\delta^2). \quad (2)$$

Public goods

We consider a density-dependent model of public goods cooperation. First, we assume that the production of public goods by an individual or group is equal to the value of its cooperation strategy, i.e. X or Y . Second, because not all of the public goods produced in a social group may be used, especially if cell density is low so that contact rate between cells and public goods molecules is low, we assume that a proportion $\exp(-kn)$ of the Y public goods produced by the group are lost; where n is the number of cells in the social groups and k is the per molecule per cell rate of uptake of public good molecules over the unit time period. Third, we assume that the growth benefit of public goods is a power function of the quantity taken up by the cell, with exponent a and scaled by an amount b , and added to a baseline growth equal to $1-b$; the exponent a describes how sustained are the fitness returns of increased access to public goods (with lower a indicating greater diminishing returns) and b describes the relative growth benefit of public goods. Finally, we assume that the production of an amount of public goods X incurs a personal multiplicative growth cost cX . Putting all this together yields the fitness function:

$$W(X, Y) = \left(1 - b + b(Y(1 - \exp(-kn)))^a \right) (1 - cX). \quad (3)$$

From expression (2), we find that the relative fitness of cheats is given by:

$$v = 1 + \frac{c}{1 - cz} \delta x - \frac{abz^{a-1}(1 - \exp(-kn))^a}{1 - b + b(z(1 - \exp(-kn)))^a} \left(\frac{1}{n} + \frac{n-1}{n} r \right) \delta x + O(\delta^2), \quad (4)$$

where z is the population average level of cooperation, and the whole-group relatedness is $R = 1/n + ((n-1)/n)r$ where r is the ‘others-only’ relatedness to the group (this is the relatedness of an individual to the others in the group – formally, the

expected relatedness of two cells randomly drawn from the social group, without replacement; Pepper 2000). Differentiating the RHS of expression (4) by n reveals the density dependence:

$$\frac{dv}{dn} = \frac{abz^{a-1}(1-\exp(-kn))^a}{1-b+b(z(1-\exp(-kn)))^a} \times \left(\frac{1-r}{n^2} - \frac{a(1-b)k}{1-b+b(z(1-\exp(-kn)))^a} \frac{\exp(-kn)}{1-\exp(-kn)} \left(\frac{1}{n} + \frac{n-1}{n}r \right) - \frac{n-1}{n} \frac{dr}{dn} \right) \delta x, \quad (5)$$

to first order in δx . Assuming $dr/dn \leq 0$, i.e. the others-only relatedness of social partners does not increase with cell density, then a sufficient condition for the relative fitness of cheats to increase with population density (positive density dependence, $dv/dn > 0$) is:

$$b \left(\frac{ak \exp(-kn)}{1-\exp(-kn)} \left(\frac{1}{n} + \frac{n-1}{n}r \right) - \left(1 - (z(1-\exp(-kn)))^a \right) \frac{1-r}{n^2} \right) > \frac{ak \exp(-kn)}{1-\exp(-kn)} \left(\frac{1}{n} + \frac{n-1}{n}r \right) - \frac{1-r}{n^2} \quad (6)$$

We now demonstrate that this is satisfied for sufficiently large values of b , and that there always exists a $b \leq 1$ that is large enough to satisfy the condition. First, if the RHS of condition (6) is positive, then the condition can be rearranged into the form $b > b^*$, where:

$$b^* = \frac{ak \frac{\exp(-kn)}{1-\exp(-kn)} \left(\frac{1}{n} + \frac{n-1}{n}r \right) - \frac{1-r}{n^2}}{ak \frac{\exp(-kn)}{1-\exp(-kn)} \left(\frac{1}{n} + \frac{n-1}{n}r \right) - \frac{1-r}{n^2} + (z(1-\exp(-kn)))^a \frac{1-r}{n^2}} \quad (7)$$

lies in the range $0 < b^* < 1$. Alternatively, if the RHS of condition (6) is negative then, if the LHS is also negative, we obtain the condition $b < b^*$ where $b^* > 1$, and if the LHS is positive we obtain the condition $b > b^*$ where $b^* < 0$; in either scenario,

the condition is satisfied irrespective of the value of b . In summary, so long as public goods are sufficiently beneficial (large enough b), the relative fitness of cheats is never a decreasing function of cell density (Figure 3.1A). For example, if the public good is necessary for growth ($b = 1$) then any density dependence in the relative fitness of cheats will be positive.

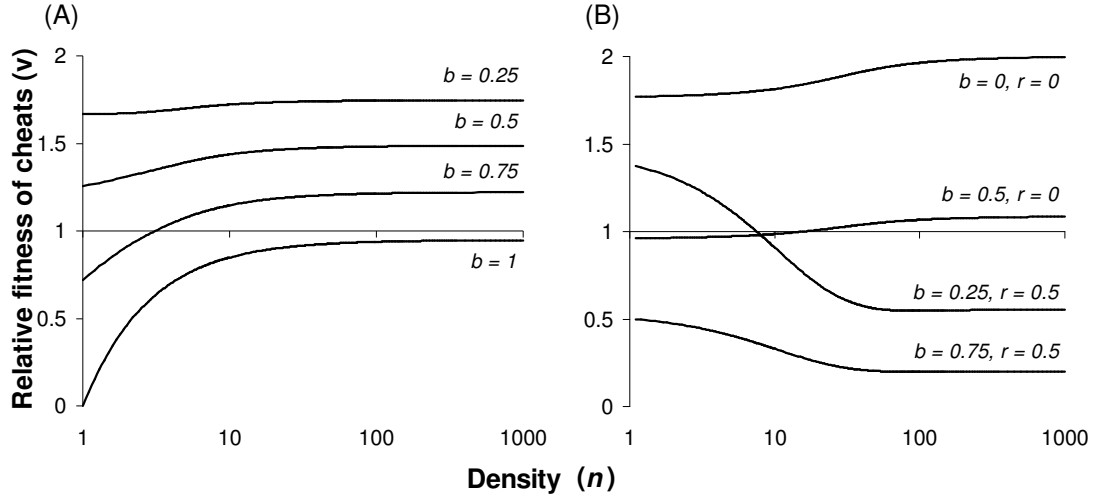


Figure 3.1. Model demonstrating how the fitness of cheats relative to cooperators can be subject to density dependence. Under public goods cooperation (A), cheat relative fitness, v , is positively related to density across most of the parameter space but is highly sensitive to b , a parameter describing how beneficial cooperation is to a recipient. For this illustration, we set $a = 1$, $c = r = 0.5$ and $k = 0.9$. We assume that cooperators invest $X = 1$ into public goods and cheats invest $X = 0$ and, mirroring the experiments, that cheats make up 10% of the population. Under self-restraint cooperation (B), cheat relative fitness can be either positive or negatively density dependent, depending on the values of both b (see above) and r , relatedness to other cells in the social group. For this illustration, we set $a = k = 0.25$, assume cooperators show self-restraint $X = 0.5$ and cheats $X = 0$, and that cheats make up 10% of the population.

We may also examine the impact of relatedness upon density dependence. Increasing the value of relatedness uniformly across all densities reduces the degree of density dependence, as shown by differentiating equation (5) with respect to relatedness.

This obtains:

$$\frac{d}{dr} \left(\frac{dv}{dn} \right) = - \frac{abz^{a-1}(1 - \exp(-kn))^a}{1 - b + b(z(1 - \exp(-kn)))^a} \times \left(\frac{1}{n^2} + \frac{a(1-b)k}{1 - b + b(z(1 - \exp(-kn)))^a} \frac{\exp(-kn)}{1 - \exp(-kn)} \frac{n-1}{n} \right) \delta x, \quad (8)$$

which is always negative. This positive density dependence we are predicting within populations is not to be confused with the negative density dependence which can arise at the metapopulation level in cases where there is a threshold benefit associated with public goods production (Brown and Johnstone 2001; Brockhurst et al. 2006; Diggle et al. 2007a).

Self-restraint

Next we consider cooperation in the form of self restraint in resource use (Frank 1996; 1998). First, analogous to the previous model, we assume that the social group of cells does not use all of its available resources but rather that a proportion $1 - \exp(-kn(1-Y))$ of resources are acquired when the group allocates effort $1-Y$ into resource acquisition (and hence it shows self restraint Y). Second, we assume that each cell acquires an amount of resource equal to its relative acquisition effort, i.e. $(1-X)/(n(1-Y))$. Finally, we assume that the conversion efficiency of a unit of resource into growth is an increasing function of self-restraint: the slower the cells take up resources, the more efficiently they metabolise those resources that are taken up (e.g. Pfeiffer et al. 2001; MacLean and Gudelj 2006; MacLean 2008). In particular, we assume that the efficiency benefit is a power function of the level of self-restraint, with exponent a and scaled by a factor b , which is added to a baseline conversion factor of $1-b$. Thus, we can write:

$$W(X, Y) = \frac{1-X}{n(1-Y)} (1 - \exp(-kn(1-Y))) (1 - b + bX^a). \quad (9)$$

From equation (2), we can write the relative fitness of cheats as:

$$v = 1 + \frac{1}{1-z} \delta x - \frac{ab}{1-b+bz} \delta x - \left(\frac{1}{1-z} - \frac{nk \exp(-kn(1-z))}{1-\exp(-kn(1-z))} \right) R \delta x + O(\delta^2), \quad (10)$$

where z is the population average level of self-restraint. Differentiating with respect to cell density n gives us:

$$\begin{aligned} \frac{dv}{dn} = & k \frac{\exp(-kn(1-z))}{1-\exp(-kn(1-z))} \left(1 - (1-z)nk \left(1 + \frac{\exp(-kn(1-z))}{1-\exp(-kn(1-z))} \right) \right) R \delta x \\ & - \left(\frac{1}{1-z} - nk \frac{\exp(-kn(1-z))}{1-\exp(-kn(1-z))} \right) \frac{dR}{dn} \delta x, \end{aligned} \quad (11)$$

to first order in δx . The total density effect is the sum of two partial effects, the first due to the impact of cell density upon resource acquisition, and the second due to the impact of cell density upon whole-group relatedness. The first effect, described by the first term on the RHS of equation (11), is always negative. The second effect, described by the second term on the RHS of equation (11), is always positive (because $dR/dn < 0$). In general, it is not possible to write a condition in terms of the model parameters that describes whether the relative fitness of cheats will increase (positive density dependence) or decrease (negative density dependence) with cell density, as this will depend on the details of how whole-group relatedness decreases with density (Figure 3.1B). While the preceding theory requires only weak selection (small phenotypic differences between cheats and cooperators), we assume stronger selection for the scenarios illustrated in Figure 3.1 and still recover the same qualitative results.

3.4. Methods

Description of strains

We replicated all our experiments with three independently-derived cooperator-cheat strain pairs in order to demonstrate that observed effects are not strain-specific (for further strain details, see Appendix A1 and Chapter 2). Briefly, these pairs were

derived from the well-categorised *Pseudomonas aeruginosa* isolates PA01 ATCC15692 (pairs 'A' and 'B') and UCBPP-PA14 (pair 'C'). While cooperators ('+') retained the siderophore production proficiency of their wild type progenitors, cheat strains ('-') had mutations conferring defective production of the primary siderophore, pyoverdine, induced, respectively, by means of ultraviolet and transposon mutagenesis (pair A; Rella et al. 1985; Hohnadel et al. 1986), deletion of the pyoverdine synthesis gene *pvdD* (pair B; Ghysels et al. 2004) or laboratory selection (pair C; Ross-Gillespie et al. 2007). In each case, cheat strains formed white colonies when grown on Kings B agar, making it possible to distinguish them from cooperator colonies, which appear green.

Experimental protocols

Our first experiment tested our model's prediction that cheat fitness would be positively correlated with overall cell density in spatially structured populations (positive density dependence). For each strain pair, we cultured + and - from freezer stock in 6 ml standard Kings B solution in 30 ml glass universal vials, incubating overnight in an orbital shaker (37°C, 200 r.p.m.). After 24 hours, the cultures were vortexed and combined to obtain a master mix culture of + and - at a volumetric ratio of 10:1.

To create conditions in which the cooperative trait (siderophore production) was important to fitness, we prepared culture media in which iron was extremely limited (CAA-agar: 5 g casamino acids, 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$ and 12 g agar L^{-1} , supplemented with 0.1 g human apo-transferrin and 1.68 g $NaHCO_3 L^{-1}$ for additional iron chelation). We set up density treatments as follows: six replicate samples of the master culture were serially diluted in M9 buffer to obtain the following dilutions: 1 in 10^6 (low density); 1 in 10^4 and 1 in 10^2 (high density). We spread 20 μl samples of these diluted cultures to 10 cm diameter plates of 25 ml CAA-agar and set these to incubate at 37 °C. We assessed, post-hoc, the actual ratio of + to - cells in this master mix by taking multiple samples, growing these overnight

on Kings B agar plates, and then counting colony-forming units (CFUs), which could be categorized as either + or - on the basis of colour and morphology.

Meanwhile, our density treatment plates were incubated at 37°C, and after 48 hours we scraped all bacterial growth from the surface of each plate with a sterile spatula and resuspended it in 6 ml M9. We vortexed and serially diluted these resuspended cultures, then plated them onto Kings B agar. We counted + and - CFUs on these plates after 24 hr incubation at 37°C, and in this way we obtained data on change in relative proportions of + and - CFUs across the competition period. We fully randomized labelling, spreading and counting of plates at each stage to minimize order effects.

Investigating the causal mechanisms underlying density dependent cheat fitness

We carried out a separate experiment to investigate the causal mechanism underlying patterns of density-dependent cheat fitness. Specifically, we tested whether the growth of cheat colonies varied with their proximity to cooperator colonies. First, we cultured strains overnight in Kings B medium then diluted them such that 1 µl of culture contained approximately 25 bacteria. Next, we set up 3 × 8 point lattices of 1 µl droplets of - culture on iron-limited agar (CAA, as above), by transferring culture from a microtitre plate using a 96-pin steel replicator tool (Boeckel Scientific). Each lattice of - droplets was bounded along one edge by an adjacent row of 8 × 1 µl droplets of a “neighbour” culture, at a density of approximately 600 cells per droplet. In this manner we obtained 36 lattices (3 strain pairs × 3 treatments × 4 replicates), each of which comprised 3 rows of 8 cheat colonies, growing on iron-limited agar, at varying distances from the row of 8 “neighbour” colonies (either +, - or M9 buffer, “0”; see Figure 3.2). We incubated plates at 37°C for 48 hrs and assayed the relative growth of cheat colonies in the lattices. First, we excised all colonies in each row, using a sterile 4 mm diameter hole-punch, and transferred the cores to an eppendorf containing 0.5 ml M9 buffer. We thoroughly vortexed each eppendorf to wash cells off the agar and into suspension, and then, taking two replicate 200 µl samples of diluent (minus agar), we estimated the cell density of the resuspended cultures by

measuring, with a spectrophotometer (M2 plate reader, Molecular Devices), their optical absorbance at 600 nm.

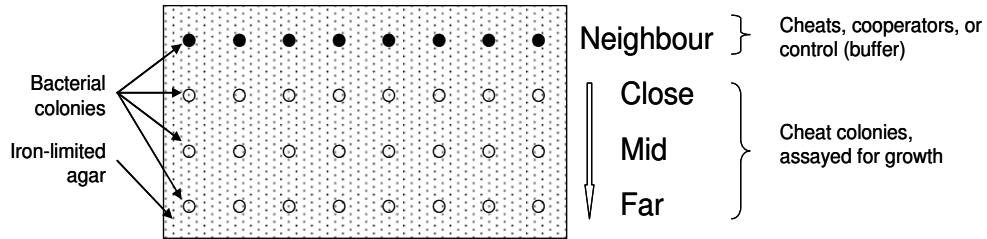


Figure 3.2. Design of experiment to demonstrate that physical proximity to cooperators is the causal mechanism underlying density dependence of cheat fitness. Circles indicate position of bacterial colonies in the lattice. Colonies from the lower three rows were assayed for growth after 48 hours.

Statistical analyses

To analyse the first set of experiments, we first calculated relative cheat fitness (v), by comparing the frequency of cheats at the beginning and end of each experiment. Specifically, v is given by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheats in the population, x_2 is their final proportion (Ross-Gillespie *et al.* 2007). The value of v therefore signifies whether cheats increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We assessed the nature and strength of the relationship between v and population density (n), using standard General Linear Models (GLMs) with n as the explanatory variable (along with *strain pair* and the interaction term, in the case of the maximal model). In each case, v and n were first log-transformed to comply with the assumptions of parametric analyses. In addition, we calculated an alternative measure for the fitness of cheats relative to cooperators, w , which is the ratio of the two strains' respective Malthusian growth parameters - essentially, the ratio of the number of doublings achieved by each sub-population (Lenski *et al.* 1991). As above, we constructed a GLM with w as the response variable, and n and *strain pair* as fixed explanatory terms (where both w and n were first log-transformed).

To analyze our productivity vs. proximity data, we first calculated the relative productivity for each sample as its optical density relative to the average optical density across all samples for that same strain pair. We then constructed a Linear Mixed Effects Model with relative productivity as the response term (transformed to $\log(x+1)$) and the following fixed effects: *strain pair* (A, B or C); *neighbour* (+, - or 0); *distance to neighbour* (close, mid or far), as well as all two-way interactions. Because data were collected for multiple levels of the *proximity* treatment from the same replicate agar plates, and were thus not independent of each other, we included *plate* as a random effect in the model. Analyses were performed using S-Plus 8.0 (<http://www.insightful.com>).

3.5. Results

In general, we found that cheats fared better under conditions of high population density (Figure 3.3). In a model including all three strain pairs, the relative fitness of cheats, v , was significantly positively correlated with initial population density ($F_{1,46} = 87.40$; $p < 0.001$). However, there were significant differences in relative cheat fitness across strain pairs (main effect: $F_{2,46} = 200.39$; $p < 0.001$) and in the patterns of density dependence across strain pairs (interaction: $F_{2,46} = 38.96$; $p < 0.001$). Indeed, when analysed separately, strain pair A (in contrast to pairs B and C) did not show significant density dependence of cheat fitness ($F_{1,16} = 0.49$; $p = 0.49$).

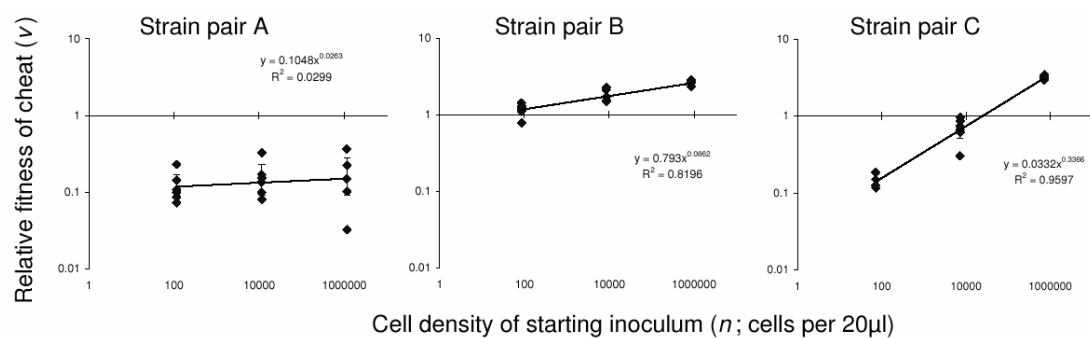


Figure 3.3. Positive density dependence of cheat relative fitness in three independently-derived strain pairs (A - C).

When we analysed our data using the alternative measure of relative fitness (w), differences between strain pairs were more pronounced. Here, strain pair A showed negative density dependence ($F_{1,16} = 12.39$; $p = 0.002$), whereas pairs B and C both showed positive density dependence, as in the above analyses. Consequently, there was no overall main effect of initial density on w ($F_{1,46} = 0.10$; $p = 0.756$), but a strongly significant interaction between strain pair and initial density ($F_{2,46} = 29.02$; $p < 0.001$). Comparing the two strain pairs that did show positive density dependence, we found further differences, in terms of the intercepts of their density dependence functions: B- had relative fitness ≥ 1 across all treatments, whereas C- had lower fitness than C+ at low cell density (v and w both < 1) but greater fitness at high density (v and w both > 1).



Figure 3.4. Interaction plot showing how the productivity of cheat colonies depends on whether or not cooperators are growing in the vicinity, and how close they are to these cooperators. Error bars show one standard error around the means.

In support of the assumption of our model, cheats were most productive when they were growing in close physical proximity to colonies of cooperators. The minimal model showed that the relative productivity of cheats was strongly influenced by the types of colonies growing nearby ($F_{2,84} = 144.04$; $p < 0.001$) and by the proximity to these colonies ($F_{2,84} = 33.88$; $p < 0.001$), although the latter did vary in strength across the three strain pairs ($F_{4,84} = 7.47$; $p < 0.001$). The effects of treatment and

proximity, and the significant interaction between them ($F_{4,84} = 46.19$; $p < 0.001$), are illustrated in Figure 3.4. There was no significant main effect of strain pair ($F_{2,9} = 1.49$; $p = 0.275$).

3.6. Discussion

In this study, we have investigated – theoretically and empirically – the conditions under which cooperation in microbes is subject to density-dependent selection. We have shown that, when considering the production of public goods, cheats are predicted to have a higher relative fitness at higher population densities (positive density dependence; Figure 3.1A). In contrast, when considering prudent use of limited resources (tragedy of the commons), cheats can have either a higher or a lower relative fitness at higher population densities (positive or negative density dependence; Figure 3.1B), depending upon the biological details. We then tested the prediction of our public goods model, using the production of iron-scavenging siderophore molecules in *Pseudomonas aeruginosa* as a model trait. As predicted, we found that cheats have higher fitness at greater host densities (Figure 3.3). Furthermore, we found support for the underlying assumption leading to this prediction, that cheats are better able to exploit the cooperative siderophore production of others, when they are physically closer to them (Figure 3.4).

We predicted that the direction of density dependent selection on cooperation depends firstly upon the form of cooperation (public good production or self-restraint in resource use) but also the finer biological details. Considering public goods, an increase in cell density will allow cheating cells a greater access to the public goods produced by its cooperative neighbours. Another way of thinking about this is that an increase in density increases the number of cells that each individual can interact with, and hence tend to decrease the relatedness of an individual to all the individuals in the group, including itself (‘whole-group’ relatedness; Pepper 2000). Both of these factors will increase the relative fitness of cheats at greater population densities. Support for this prediction has been provided by Greig and Travisano (2004) in relation to invertase production in yeast, and this study in relation to siderophores in

bacteria (Figure 3.3). Greig and Travisano (2004) suggested that fluctuating selection for increased then decreased cooperation, as yeast cells colonise new patches and then grow to high densities, could allow for the maintenance of both cooperators and cheats in the population (polymorphism). More generally, we might expect microbes to facultatively adjust their cooperation strategies conditionally upon local cell densities, for example through the use of quorum sensing systems (Brown and Johnstone 2001; Keller and Surette 2006; Diggle et al. 2007a, b).

In contrast, when considering cooperative self-restraint in resource uptake, the relative fitness of cheats, which have a higher rate and less efficient metabolism of resources, can either increase or decrease with host density, depending upon biological details (parameter values). Whilst the advantage of more efficient metabolism of resources is greater when cell density is high enough for resources to be growth-limiting, the individual incentive for such cooperation can decrease with increasing cell density if the whole group relatedness of interacting cells declines with increasing number of social partners. These two effects are in opposition and so cheats that utilise resources quickly and inefficiently may be increasingly favoured or disfavoured as cell density increases, depending upon which of the two effects is strongest. The prediction of cheats having a lower fitness at higher densities (negative density dependence) has previously been made in theoretical studies, and is supported by empirical studies of the competition between yeast strains following alternative metabolic strategies (Pfeiffer and Bonhoeffer 2003; MacLean and Gudelj 2006; MacLean 2008). In yeast, respirers (cooperators) utilise resources slowly but with a higher yield of ATP than respiro-fermenters (cheats), which enjoy fast resource use but with a much smaller ATP yield. Whilst the observed negative density dependence of cheat fitness in yeast may occur for the reason discussed above, an added complication in this system is that the metabolic pathway employed by cheats yields toxic byproducts which decrease fitness as they accumulate, and this accumulation of toxins is increased at higher population densities (MacLean and Gudelj 2006). Thus, one consequence of toxin accumulation in this system is that it increases the parameter space over which negative density dependence is expected. Another potential complication not considered here is the possibility of interactions

between self-restraint and public goods cooperation, when they are linked (MacLean 2008).

Patterns of density-dependent fitness are intrinsically sensitive to the measure of fitness used. Our preferred measure of relative fitness (v , see above) captures the relative change in frequency of a genotype over time and, as such, is compatible with the population genetics concept of fitness. However, while a change in genotype frequency over time can indeed reflect different rates of growth or persistence among genotypes *within* a given population, this measure can be problematic in between-population comparisons if the extrinsic conditions (i.e. treatments) afford different populations different opportunities for growth. When resources are finite and fixed (i.e. batch culture, as in our experiment), populations starting from low density will complete more generations during the observation period because they start growing at a density far below carrying capacity. In contrast, at high density a population will undergo little such exponential growth before reaching stationary phase. Thus, in the low inoculum scenario, absolute fitness differences between competing strains could be magnified relative to what would be seen in populations starting from high density inocula, resulting in density dependent fitness effects – that may have nothing to do with social interactions among strains. We investigated this possibility by also considering an alternative fitness measure that controls for differences in the number of generations (w , see above). Analyses of strain pairs B and C yielded qualitatively similar results for both fitness measures, confirming that the density-dependent fitness patterns we observed in these strains (Figure 3.3) cannot be explained by this confounding generation effect only.

Our experimental data on siderophore production in *Pseudomonas aeruginosa* provided support for the prediction of our public goods model in two out of the three independent strain pairs we tested. In these two cases, relative cheat fitness was positively correlated with population density (Figure 3.3). Across all three of the pairs, however, there was considerable variation in the intercepts of the density dependent fitness function. One cheat strain consistently underperformed its cooperator progenitor (pair A) under our experimental conditions; another showed

greater relative fitness at all densities tested (pair B); while in the third, experimentally evolved pair, cheats outperformed cooperators at high density but lost the advantage at low density (pair C). How can we account for these differences? Our theoretical model for public goods cooperation (Figure 3.1A) predicts that the degree to which cooperation benefits recipients, b , can influence the shape, and in particular, the intercept of the relationship between density and cheat fitness. Where cooperation is strongly beneficial, cheats show low relative fitness values ($v < 1$) across a range of n , whereas if the benefits of cooperation are more marginal, cheat fitness may be greater than 1 across all densities. We postulate that differences in this parameter may, at least in part, underlie the different patterns of density dependence we see across our strain pairs (Figure 3.3). For instance, B- differs from B+ only at the *pvdD* locus (hence, marginal b), whereas in strain pair A, b may be larger because A- is also defective for additional traits involved in iron acquisition (e.g. production of pyochelin and quinolones; Steve Diggle, unpublished data). Similarly, strain C- could be acting as a cheat not only for siderophore production, but for other traits too. This highlights the value, when studying the fitness consequences of a given trait, of testing multiple, independently-derived bacterial strains (Velicer et al. 2000; Ross-Gillespie et al. 2007). Gene deletions, for instance, could give rise to unforeseen pleiotropic effects, whereas artificial selection against a given trait could generate more ‘natural’ mutants, with compensatory adaptations to counteract potential negative pleiotropic effects associated with the loss of the target trait.

Whilst we have focused on microbes, our predictions could also apply to other taxa. The importance of density dependence in the production of public goods has also been much discussed for humans, in the economics literature. Since Olson (1965), a number of economics studies of public goods dilemmas have noted how cooperative behaviour frequently decreases and “free-rider” behaviour increases within larger groups (e.g. Andreoni 1988; Chamberlin 1974; Gaube 2006; Stonebraker 1993). However, as is typically the case with human behaviour, even small changes in experimental design or context can have important effects. For instance, Isaac and Walker (1988) and Isaac et al. (1994) showed that when the marginal incentives to individuals were scaled to remain constant across different group sizes, free-riding

behaviour actually decreased at higher densities. More generally, for any organisms that live at high density, cooperation will be most favoured when interactions take place between close relatives (i.e. cooperators interact almost exclusively with other cooperators; Hamilton 1964). A common way to achieve this is to have relatively low dispersal into or out of the population – a pattern that has been observed in both microbes (e.g. biofilms, where cells encase themselves in a viscous matrix; Xavier and Foster 2007) and metazoans, including social invertebrates (Bilde et al. 2005; Hamilton 1972; Peer and Taborsky 2007) and molerats (Jarvis *et al.* 1994).

4. Social conflict over siderophore production and its consequences for bacterial pathogenesis

4.1. Summary

Co-infections can have quite different fitness consequences for pathogens and hosts depending on how co-infecting strains interact. In the simplest case, scramble competition could favour rapid exploitation and consequently high virulence; whereas, if pathogens cooperate to infect a host, co-infection could introduce social cheats which selfishly undermine this cooperation and hence reduce the virulence. We investigated the latter scenario using the cooperative production of siderophores by the bacterium *Pseudomonas aeruginosa* in experimental infections with an insect (*Galleria mellonella*) and a plant (*Lactuca sativa*) host. In contrast with previous studies, we found no evidence for a fitness benefit of siderophore production *in vivo* and, consequently, no evidence that non-producing strains gained relative benefits through exploiting producers in co-infection. We did, however, observe attenuation of virulence in some infections with siderophore-deficient strains. More generally, we review evidence for the importance of bacterial siderophores in infection contexts and discuss the limitations of using experimental infection models to study this trait.

4.2. Introduction

Why are some infections so harmful while others are comparatively benign? Much attention has been focused on the evolution of virulence (damage to host; Bull 1994; Ewald 1994; Frank 1996; Day and Burns 2003), but one area in which existing theory does not adequately account for observed patterns concerns situations in which multiple co-infecting agents interact (Gandon et al. 2001; Read and Taylor 2001). Classic virulence theory predicts that growth of a pathogen is traded off against its transmission efficiency, because healthier, longer-lived hosts provide more opportunities for transmission (Frank 1996). Thus, selection could favour pathogen “restraint” and lower virulence (Frank 1998). Under multiple infection, however, scramble competition among co-infecting strains favours rapid host exploitation and therefore higher virulence – a classic tragedy of the commons

situation (Hardin 1968). Thus, conflict between co-infecting pathogens, and their resultant virulence, should correlate negatively with kinship among the strains (Hamilton 1972; Bremermann and Pickering 1983; Frank 1992; Frank 1994; Nowak and May 1994; van Baalen and Sabelis 1995; Frank 1996) but so far, empirical support for this prediction has been limited and ambiguous (Herre 1993; Herre 1996; Taylor et al. 1998; Chao et al. 2000; Read and Taylor 2001; Davies et al. 2002; De Roode et al. 2003; Massey et al. 2004; Hodgson et al. 2004).

In some cases at least, the specific underlying biology of the host and/or the pathogen could give rise to different relationships (Ganusov and Antia 2003). For example, hosts might deploy different immune responses against single versus multiple infections (Taylor et al. 1998) which obscure any adaptive responses of the pathogens themselves to the different infection types. Alternatively, if we allow for the possibility of cooperative interactions among pathogens, we can obtain quite different predictions for the relative virulence of co-infections versus single infections (Gardner et al. 2004; Buckling and Brockhurst 2008). Here we test the idea that when pathogens rely on cooperation to invade and exploit their hosts, high relatedness within an infecting population will favour more cooperation and thus lead to more, rather than less virulent infections (Chao et al. 2000; Brown et al. 2002; West and Buckling 2003). This scenario may be especially relevant among microbial pathogens, given the striking diversity and ubiquity of cooperative behaviours we now recognize to occur in such populations (Crespi 2001; West et al. 2006; West et al. 2007). Probably the most common form of cooperation involves the production of public goods – extracellular products which, while metabolically costly to the producing cell, confer general benefits at the local population level (West et al. 2007). Such products include digestive enzymes; anti-competitor toxins; antibiotic degrading enzymes; protective biofilm polymers; surfactants to facilitate movement; and a wide variety of virulence factors which inactivate immune defenses and provide access to host resources.

Among the best-characterised bacterial public goods are siderophores, iron scavenging molecules produced by cells in response to iron starvation. Bacteria, like

other organisms, require iron for redox-dependent metabolism but in most environments, soluble iron (Fe(III)) is scarce or, in the case of pathogens residing within a host, actively withheld using proteins of high iron-affinity (Ratledge and Dover 2000; Andrews et al. 2003; Wandersman and Delepelaire 2004; Ong et al. 2006). Bacteria have evolved a number of strategies to overcome this problem. Foremost among these is the secretion of siderophores, which chelate ferric ions in the environment, making them available for uptake by the bacteria as a ferrisiderophore complex (Guerinot 1994; Braun 1995; Schalk 2008). To date, more than 500 siderophores have been identified, from most bacterial genera (Wandersman and Delepelaire 2004; for fungal siderophores see Haas et al 2008).

A well-studied model for siderophore production is the Gram-negative proteobacterium, *Pseudomonas aeruginosa* (Visca et al. 2007). This species is an opportunistic pathogen, present in most terrestrial and aquatic habitats, and capable of causing infection in a range of plants and animals (D'Argenio 2004). In humans, it is an important nosocomial pathogen and a potentially serious threat to immune-compromised patients, establishing infections in eyes, burn wounds, urinary tracts and around implanted devices, and especially in respiratory tissue (Lyczak et al. 2000; Lyczak et al. 2002). It is also the primary pathogen in cystic fibrosis lung infections, where it gives rise to chronic and notoriously persistent infections (Harrison 2007). The majority of *Pseudomonas* species produce the yellow-green siderophore pyoverdine (Ravel and Cornelis 2003; Visca et al. 2007) which, in the case of *P. aeruginosa*, is held to be the primary means of iron acquisition (Ankenbauer et al. 1985; Vasil and Ochsner 1999; Visca et al. 2007).

Because of its importance for bacterial growth, siderophore production is expected to contribute to virulence *in vivo*. Consistent with this idea, there is accumulating evidence of pyoverdine synthesis by *P. aeruginosa* during pulmonary infection (Haas et al. 1991; Handfield et al. 2000; Hunt et al. 2002; Palmer et al. 2005) and, in several different animal models of disease, pyoverdine-defective mutants show a significantly reduced ability to cause infection (Table 4.1). Infection models using other bacteria also demonstrate the involvement of siderophores in virulence, in

Vibrio (Wolf and Crosa 1986; Litwin et al. 1996), *Yersinia* (Bearden et al. 1997), and *Erwinia* (Expert 1999). Apart from its role in growth, uptake of iron by pyoverdine may, in turn, stimulate other virulence genes (Lamont et al. 2002; Beare et al. 2003) and there is also evidence to suggest that it is required for biofilm formation (Banin et al. 2005; Patriquin et al. 2008).

We have argued (above, and previously West and Buckling 2003; Griffin et al. 2004; Ross-Gillespie et al. 2007) that siderophores such as pyoverdine are public goods, and that their production is a cooperative behaviour. As such, social evolution theory predicts that siderophore production could be vulnerable to exploitation by “cheats” that do not contribute siderophores of their own. *In vitro* experiments have shown that pyoverdine-defective mutants (cheats) do not grow well in iron-limited conditions but are able to use pyoverdine produced by pyoverdine-proficient strains (cooperators) when grown in mixed culture. Since cheats avoid the metabolic costs of pyoverdine production, they can achieve higher relative fitness than cooperators, even to the detriment of population-level productivity (Griffin et al. 2004; Ross-Gillespie et al. 2007). Pyoverdine-defective cheats are frequently isolated from clinical settings (Huston et al. 2004), including cystic fibrosis lungs (De Vos et al. 2001) where, evidence suggests, they may gradually accumulate over time (Smith et al. 2006). Moreover, both clinical *P. aeruginosa* infections and environmental populations frequently harbour multiple genetically distinct strains (Mahenthiralingam et al. 1994; Govan and Deretic 1996; Burns et al. 1998; Pirnay et al. 2005; Smith et al. 2006; Fothergill et al. 2007), so there is good reason to suppose that cooperator-cheat dynamics could naturally occur.

What then are the potential consequences of these social dynamics for virulence? A key theoretical prediction is that the presence of siderophore cheats in a mixed infection should decrease virulence relative to a wild type infection (West and Buckling 2003). An earlier study with a *Vibrio*-Salmonid model confirmed this result (Wolf and Crosa 1986), and was further able to demonstrate that cheats had low fitness when growing on their own, but grew much better in co-infection with siderophore-producing cooperator strains, to the extent that they greatly increased

their relative proportion of the mixed population during the course of the infection. Working with an insect host (the greater waxmoth, *Galleria mellonella*), Harrison et al. (2006) obtained qualitatively equivalent results with *P. aeruginosa* pyoverdine cheats. Such patterns contrast strongly with those predicted by classical theory which assumes scramble competition among co-infecting strains (Frank 1996) yet, to our knowledge, these two studies are the only *in vivo* tests of this theory to date. In this study, therefore, we attempted to replicate and build on this work in two ways. First, we consider an additional host model (lettuce). Secondly, we consider multiple pairs of *P. aeruginosa* strains. In their experiments, Harrison et al. (2006) competed two strains that differed from each other at multiple loci and for multiple traits besides simply siderophore production. Here we use three independent cooperator-cheat pairs, derived from different clonal backgrounds using multiple methods - including genome-wide mutagenesis, artificial selection, and targeted gene disruption.

4.3. Methods

Bacterial strains and model hosts

We used three *Pseudomonas aeruginosa* strain pairs in our infection experiments, matching those we used previously for *in vitro* experiments (Ross-Gillespie et al. 2007; chapters 2 and 3; appendix A1). Briefly, we derived strain pairs ‘A’ and ‘B’ from the well-categorised wild-type isolate PA01 ATCC15692 and pair ‘C’ from strain UCBPP-PA14. While cooperators (‘+’) exhibited wild-type levels of siderophore production; cheat strains (‘-’) were defective for production of the primary siderophore, pyoverdine. Mutations for this trait were induced by means of ultraviolet and transposon mutagenesis (pair A; Rella et al. 1985; Hohnadel et al. 1986), deletion of the pyoverdine synthesis gene *pvdD* (pair B; Ghysels et al. 2004) or laboratory selection (pair C; Ross-Gillespie et al. 2007). In each case, cheat colonies were phenotypically distinguishable from cooperator colonies when grown on Kings B agar on the basis of differential pigmentation. We performed infections with both an insect and a plant host (Figure 4.1). For the insect host, we used larvae of the greater waxmoth (*Galleria mellonella*; Livefood UK;

<http://www.livefoods.co.uk>). As final instar larvae, waxmoths did not feed, and under refrigeration maintained good body condition for up to a week following purchase. Our methodology is derived from a model developed by Lysenko (1963), Jander et al. (2000) and, especially, Harrison et al. (2006). For the plant host, we obtained commercially-grown Romaine lettuces (*Lactuca sativa*) from a local supermarket, and infected them within hours of purchase. This lettuce infection model, on which we based our methodology, has been widely used to study *P. aeruginosa* pathogenesis (e.g. Rahme et al. 1997; Filiatrault et al. 2006; Vives-Florez and Garnica 2006).

Preparation of bacterial inocula

We conducted infection experiments one strain pair at a time, with at least two blocks per pair. For each pair, we grew monocultures of + and - from freezer stock overnight, using 6 ml of standard Kings B medium in an orbital shaker (37°C, 200 r.p.m.). After 24 hours, we prepared our treatment inocula. First, we twice pelleted cells by centrifugation (3 min; 6000 r.p.m.) and washed them in buffer (0.8% NaCl solution in the case of waxmoth infections; 10 mM MgSO₄ in the case of lettuce infections). We set up three treatments: pure cooperator ('+'); pure cheat ('-'); a mix of + and - ('M'; at a volumetric ratio of 1:1 in the case of the waxmoth model or 3:1 in the case of lettuce model) as well as a control treatment, ('0'; buffer only, as above). We injected waxmoths with a 10 µl infecting dose of $4-6 \times 10^3$ cells; whereas, for lettuces we used a more concentrated dose of $3-4 \times 10^7$ cells. In either case, we administered the dose using a 10 µl precision syringe (<http://www.hamiltoncompany.com>) fitted with a 26S gauge needle, which was cleaned between each injection using 100% EtOH and a sterile water rinse.

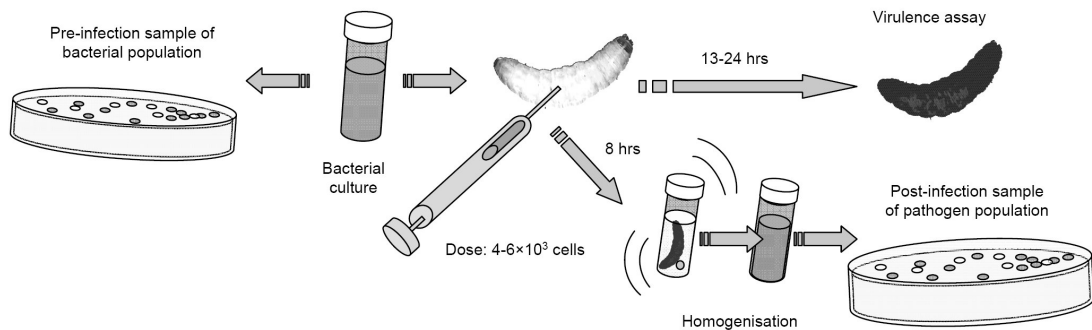
Infection protocols

(a) Waxmoth model

We conducted separate assays for virulence and bacterial growth, although in either case we performed the initial infections in the same way. In the case of virulence

assays, for each strain pair in turn, we randomly allocated replicates to treatments split equally across two blocks. For bacterial growth assays, meanwhile, we obtained data from one single-block experiment per strain pair. For each larva, we weighed it to the nearest 0.1 mg, swabbed it with 70% ethanol to kill surface contaminants, and injected it in the abdomen between the first prolegs. Following injection, we randomly distributed larvae to individual wells of 24-well sterile microtitre plates and incubated them at 37°C. Finally, we took samples of the inocula, plated them to Kings B agar and, following overnight incubation at 37°C, counted colony-forming units (CFUs) to obtain estimates of initial cell densities and starting ratios of cooperators to cheats.

(A) WAXMOTH INFECTION MODEL



(B) LETTUCE INFECTION MODEL

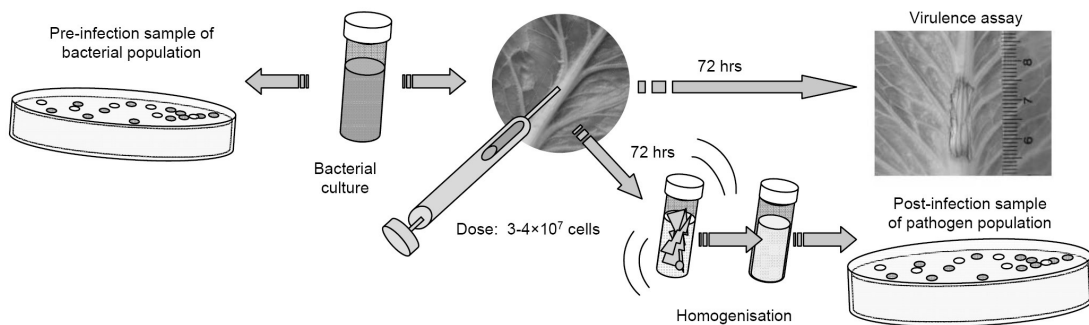


Figure 4.1. Experimental protocols used in (A) waxmoth and (B) lettuce infection models.

For virulence assays, we monitored mortality at hourly intervals between 13 and 24 hours post-inoculation. We scored larvae as dead if they failed to respond to mechanical stimulation of the head and prolegs. Distinct melanism was also evident in dead larvae (Figure 4.1A). Control treatment larvae showed negligible mortality.

For bacterial growth assays, we retrieved larvae after 8 hr incubation (as per Harrison et al. 2006), and homogenized them individually in 0.5 ml 0.8% NaCl by rapid shaking with a sterile ceramic bead (FastPrep FP120; <http://www.mpbio.com>; 4 m/s for 2 min). We centrifuged the homogenates at low speed (1000 r.p.m.; 3 min) to separate host tissues into distinct bands, then took samples from the liquid (haemolymph) fractions and after appropriate serial dilution in 0.8% NaCl, spread them using automated spiral plating (EddyJet; <http://www.iul-inst.com>) to plates of Kings B agar supplemented with 15 µg/ml ampicillin to restrict the growth of non-target bacteria (this concentration does not impede growth in *Pseudomonas aeruginosa*). We then incubated these plates overnight at 37°C and counted CFUs to obtain estimates of the endpoint cell densities achieved by the infecting populations and, for mixed infections, the endpoint ratios of cooperators to cheats. When estimating the final pathogen population density within a given larva, we had to consider how size differences among larvae could affect the total volume of homogenate from which we drew our sample. In a separate experiment (data not shown), we regressed larval mass against the volume of liquid larvae displaced, thereby establishing that larval volume (ml) = $1.032 \times \text{larval mass (g)}$. We applied this correction factor to each homogenized host such that our estimates of final pathogen density were for the complete host (i.e. the entire infecting population), rather than per unit of host tissue.

(b) Lettuce model

From each lettuce, we selected four leaves of similar size (one per treatment), which we washed briefly with 0.1% bleach to kill surface contaminants and then patted dry with sterile tissue. We cut 50 mm diameter discs from the centre of the leaves using a cookie cutter, and placed them individually in 10 cm petrie dishes. We then inoculated leaf discs with bacteria by injection into the midrib using a 10 µl RN Hamilton syringe fitted with a 26S needle. Finally, we placed the leaf discs, in their individual petrie dishes, in a sealed container kept humid by the addition of filter papers moistened with 10 mM MgSO₄ and incubated them at room temperature on the laboratory bench. We took samples of the inocula, diluted them, and plated them to Kings B agar. Following overnight incubation at 37°C we counted the CFUs

evident on these plates to obtain an estimate of inoculum densities and, in the case of the mixed treatment, an estimate of the starting ratio of cooperators to cheats.

After 72 hours incubation, we assayed virulence by measuring the maximum extent of tissue damage along the midrib of the leaf disc, taking our measurements post-hoc from scale-standardized digital photographs (Figure 4.1B). Control leaf discs, injected with MgSO_4 buffer only, showed negligible tissue damage. We then suspended whole leaf discs in 2 ml 10 mM MgSO_4 and homogenized them (FastPrep FP120; 4 m/s for 2 min). We took samples of the homogenate and, after serial dilution, spread them to plates of Kings B agar supplemented with 15 $\mu\text{g/ml}$ ampicillin. We incubated these plates overnight at 37°C then counted CFUs to obtain estimates of the endpoint cell densities achieved by the infecting populations and, for mixed infections, the endpoint ratios of cooperators to cheats.

Statistical analyses

We analysed waxmoth mortality data using parametric survival analysis. We specified a Weibull error structure, which, because it does not assume a constant probability of death through time, is commonly used in survival analysis (Carroll 2003). The maximal model included a blocking factor but, since it had no significant effect, it was removed from the final model. We defined bacterial growth as the number of doublings an infecting population underwent during the observed period of infection, and calculated this for each host as per Lenski et al. (1991). In the case of mixed infections (treatment ‘M’), we were also able to estimate, separately, the growth of the cooperator and cheat subpopulations, since we had estimates for total population size and proportion of each strain type at both the start and end points of the infection period. For either infection model, we analysed bacterial growth data with linear mixed effects models or, where appropriate, ANOVA. In particular, since lettuce infections involved multiple replicate blocks per strain pair (with treatments equally represented in each), we began our analyses with maximal models including the random effects *block* and, nested within it, *lettuce identity*. Where these random effects comprised a substantial portion of the total error variance (>5%) they were

retained in the model, but in other cases, they were excluded and the resulting minimal model was then a 1-way ANOVA. Finally, for each mixed infection, we calculated values for the relative fitness of cheats (v) by comparing the frequency of cheats at the beginning and end of each infection. Specifically, v is given by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheats in the inoculum and x_2 is their final proportion (Ross-Gillespie et al. 2007). The value of v therefore signifies whether cheats increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We tested whether v differed significantly from 1 using a Z-test. All analyses were performed in S-Plus 8.0 (<http://www.insightful.com>).

4.4. Results

(a) Waxmoth model

For two out of three strain pairs tested, we observed virulence patterns that accorded with our theoretical predictions and with previous results (Harrison et al 2006): waxmoths infected with siderophore-deficient bacterial strains survived relatively longer than those infected with wild type strains, while co-infections resulted in intermediate virulence (Figure 4.2A). Surprisingly, however, this pattern of virulence held only for the strains derived from a PA01 background (Parametric survival; pair A: $X^2 = 10.6$, d.f. = 2, $n = 103$, $p = 0.005$; pair B: $X^2 = 10.8$, d.f. = 2, $n = 84$, $p = 0.005$), whereas for pair C, derived via laboratory selection from a PA14 background, the opposite pattern was evident ($X^2 = 13.1$, d.f. = 2, $n = 90$, $p = 0.001$). Unlike Harrison et al (2006) we were unable to find a correlation between infection virulence and bacterial growth rates (Figure 4.3A). In each case, the growth rate of the total bacterial population did not differ significantly across different infection types (ANOVA; Pair A: $F_{2,24} = 2.494$, $p = 0.104$; Pair B: $F_{2,24} = 0.107$, $p = 0.899$; Pair C: $F_{2,24} = 0.329$, $p = 0.723$). Moreover, we did not find any evidence to suggest that cheats gained a fitness advantage when in co-infection with cooperators as compared with single infection conditions (Figure 4.3A; ANOVA; Pair A: $F_{1,18} = 0.026$, $p = 0.875$; B: $F_{1,18} = 3.276$, $p = 0.087$; C: $F_{1,18} = 1.508$, $p = 0.236$). Finally, the relative fitness, v , of cheats in co-infection (i.e. relative to cooperators in co-

infection) was, in each case, significantly less than 1, indicating that cheats were actually at a disadvantage compared with cooperators under the conditions tested (1-tailed Z-test; Pair A: $v = 0.384$; $t = -14.655$, d.f. = 9, $p < 0.001$; Pair B: $v = 0.814$; $t = -8.400$, d.f. = 9, $p < 0.001$; Pair C: $t = -24.718$, d.f. = 9, $p < 0.001$).

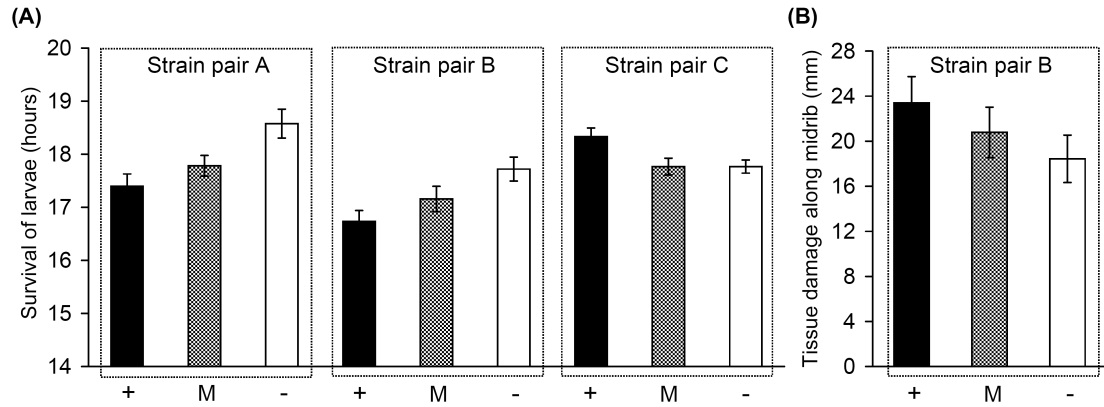


Figure 4.2. Variation in virulence across different pathogen strain pairs and infection types for (A) waxmoth and (B) lettuce infection models. Black bars indicate infections with cooperator strains (+) strains only; white bars indicate infections with cheat strains (-) only; and checked bars indicate co-infections with both cooperators and cheats (M). Error bars indicate one standard error.

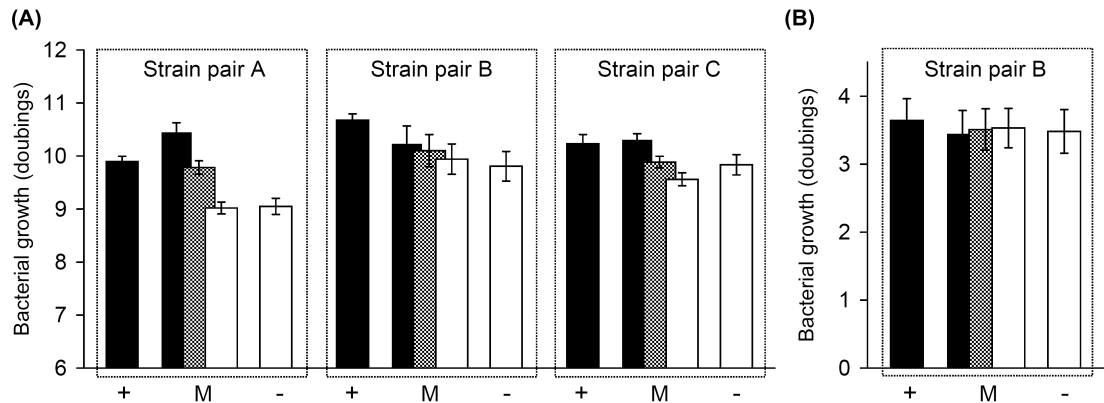


Figure 4.3. Variation in bacterial growth across different strains and infection types for (A) waxmoth and (B) lettuce infection models. Black bars refer to populations of cooperator cells, either growing alone (+) or in a co-infection (M). White bars refer to populations of cheat cells, either growing alone (-) or in a co-infection (M). Checked bars refer to the overall growth of a mixed population of cooperators and cheats. Error bars indicate one standard error.

(b) Lettuce model

We found no evidence for an effect of infection type on virulence in our lettuce infection model (Figure 4.2B). Similar levels of tissue damage were observed for infections resulting from, respectively, the PA01 wild type strain (+), a *pvdD*-knockout cheat strain (-), or a mixture of the two strain types (LMER: $F_{2,38} = 0.3694$; $p = 0.6936$). Similarly, we observed no significant difference in overall bacterial growth across the three treatments (Figure 4.3B; ANOVA: $F_{2,63} = 0.0759$, $p = 0.9270$) nor did we find any evidence for a growth advantage for cheats in co-infection as compared to single infection (Figure 4.3B; ANOVA: $F_{1,42} = 0.1660$; $p = 0.6860$). Finally, mean relative fitness, v , of cheats in co-infection (i.e. relative to cooperators in co-infection) was not significantly greater than 1 (1-tailed Z test: $t = 0.8085$, d.f. = 21, $p = 0.2139$), suggesting that cheats had no advantage over cooperators under the conditions tested.

4.5 Discussion

We tested the hypothesis that social conflict among co-infecting pathogens could affect their fitness and hence the resulting virulence of an infection but we found no evidence, in either of the models we considered, to suggest that cheating by siderophore-defective mutants has important consequences for bacterial fitness *in vivo*. Cheats grew equally well in single infections as they did in co-infection and overall bacterial growth rates did not differ significantly across infection treatments (Figure 4.3). Despite this similarity in bacterial growth across treatments, we did find, in the waxmoth model at least, that virulence varied across infection types, though not always in the expected direction (Figure 4.2). Our study, which extended previous empirical work by considering multiple pathogen strain pairs and multiple host models, produced results that contrast with those of these earlier studies (Wolf and Crosa 1986; Harrison et al. 2006), both of which indicated that siderophore cheats can exploit cooperators in a co-infection context, and that pathogen productivity, and hence virulence, are consequently attenuated in such cases. Similarly, our results contrast with experiments where iron-limitation conditions were simulated *in vitro* (Griffin et al. 2004; Ross-Gillespie et al. 2007).

Pyoverdine production is widely considered to play a central role in iron-metabolism and pathogenicity in *P. aeruginosa* (Visca et al. 2007) and in light of the results from recent competition experiments (Griffin et al. 2004; Ross-Gillespie et al. 2007) it has been hailed as a classic example of a public-goods cooperative trait in the growing field of sociomicrobiology (West et al. 2006; Buckling et al. 2007; West et al. 2007). Why then, in the present study, did we not observe fitness differences between strains or evidence of cheating? Evidently, the explanation is that under the particular conditions we tested, bacteria were able to obtain sufficient iron from the host environment irrespective of whether or not they had recourse to pyoverdine. This could arise if either (a) the hosts we used did not impose sufficiently iron-limited conditions on the bacteria infecting them, at least not over the short time scales we considered, or (b) the bacteria employed alternative mechanisms for iron acquisition, and thus did not need pyoverdine.

Considering (a), the first question is whether the host models we employed are appropriate for this particular question. The importance of iron to *P. aeruginosa* metabolism, and the role of siderophores in iron acquisition have both been well-documented *in vitro* (Visca et al 2007), and mutant strains defective for siderophore production have been shown to have attenuated virulence in several rodent models (Table 4.1). However, to date, relatively little attention has been focused on the role of siderophores in non-mammalian infection models (Table 4.1; Haas et al. 2008), which is especially surprising given the widespread interest in using alternative host models to identify conserved mechanisms of pathogenesis in wide-spectrum pathogens like *P. aeruginosa* (Rahme et al. 1995; Rahme et al. 1997; Rahme et al. 2000; Donabedian 2003). The few data that are available suggest that siderophores may be less important in non-mammalian hosts, possibly because such hosts have less efficacious systems of iron sequestration and thus provide bacteria with relatively easier access to iron (Ong et al 2006). In our experiments, we used the Romaine lettuce varietal for the purposes of consistency and comparison with other studies of *P. aeruginosa* pathogenicity (e.g. Rahme et al. 1997; Filiatrault et al. 2006; Vives-Florez and Garnica 2006). However, even compared with other lettuces, this is a relatively iron-rich organism (USDA 2007).

Table 4.1. Evidence from experimental infections for the effects of pyoverdine production on virulence

Host organism	Infection model	Control strain	Siderophore-defective mutant strain		Effect on virulence (relative to control strain)	Reference
			Strain identity	Mutation type		
PLANTS						
<i>Populus trichocarpa</i>	Root colonization	PA01	PA2663-UW	targeted mutation of <i>ppyR</i> ^a	No difference	Attila et al. 2008b
<i>Hordeum vulgare</i>	Seed germination inhibition	PA01	PA2663-UW	targeted mutation of <i>ppyR</i> ^a	Impaired	Attila et al. 2008b
<i>Medicago sativa</i>	Injured root tips	FRD1	FRD-SS161	targeted mutation of <i>pvdS</i> ^b	No difference	Silo-Suh et al. 2002
<i>Lactuca sativa</i>	Leaf infiltration	PA01	PAO6609	non-targeted mutagenesis	No difference	This study
INSECTS						
<i>Galleria mellonella</i>	Abdominal injection (larvae)	PA01	PAO6609	non-targeted mutagenesis	Impaired	Harrison et al. 2006
<i>Galleria mellonella</i>	Abdominal injection (larvae)	PA01	PA01Δ <i>pvdD</i>	targeted mutation of <i>pvdD</i> ^c	Impaired	This study
<i>Galleria mellonella</i>	Abdominal injection (larvae)	PA01	PAO6609	non-targeted mutagenesis	Impaired	This study
<i>Galleria mellonella</i>	Abdominal injection (larvae)	7F+	7K-	laboratory selection	No difference	This study
MAMMALS						
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PA01	PA01Δ <i>pvdS</i>	targeted mutation of <i>pvdS</i> ^b	Impaired	Wilderman et al. 2001
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PA01	PAO9020	targeted mutation of <i>pvdI</i> ^d	Impaired	Lehoux et al. 2000
<i>Mus musculus</i>	Intraperitoneal (acute)	PA01	PAO9020	targeted mutation of <i>pvdI</i> ^d	Impaired	Lehoux et al. 2000
<i>Mus musculus</i>	intramuscular (immunosuppressed)	PA01	PAD06	targeted mutation of <i>pvdA</i> ^e	No difference	Takase et al. 2000
<i>Mus musculus</i>	intramuscular (immunosuppressed)	PA01	PAD07	targeted mutation of <i>pvdA</i> and <i>pchD</i> ^{e,f}	Impaired	Takase et al. 2000
<i>Mus musculus</i>	intranasal (immunosuppressed)	PA01	PAD06	targeted mutation of <i>pvdA</i> ^e	No difference	Takase et al. 2000
<i>Mus musculus</i>	intranasal (immunosuppressed)	PA01	PAD07	targeted mutation of <i>pvdA</i> and <i>pchD</i> ^{e,f}	Impaired	Takase et al. 2000
<i>Oryctolagus cuniculus</i>	aortic endocarditis	PA01	PA01Δ <i>pvdS</i>	targeted mutation of <i>pvdS</i> ^b	Impaired	Xiong et al. 2000
<i>Oryctolagus cuniculus</i>	aortic endocarditis	PA01	PA01Δ <i>pvdS</i>	targeted mutation of <i>pvdS</i> ^b	Impaired	Xiong et al. 2000
<i>Oryctolagus cuniculus</i>	tricuspid endocarditis	PA01	PA01Δ <i>pvdS</i>	targeted mutation of <i>pvdS</i> ^b	No difference	Xiong et al. 2000
<i>Mus musculus</i>	Burn (acute)	PAO6049	PAO6609	non-targeted mutagenesis	Impaired	Meyer et al. 1996
<i>Mus musculus</i>	Burn (acute)	PAO6049	PAO6606	non-targeted mutagenesis	Impaired	Meyer et al. 1996
<i>Mus musculus</i>	Burn (acute)	PA01	PAO6609	non-targeted mutagenesis	Impaired	Meyer et al. 1996
<i>Mus musculus</i>	Burn (acute)	PA01	PAO6606	non-targeted mutagenesis	Impaired	Meyer et al. 1996
<i>Mus musculus</i>	Burn (acute)	PA01	PAO6616	non-targeted mutagenesis	Impaired	Meyer et al. 1996
<i>Mus musculus</i>	Burn (acute)	PA01	PAO6622	non-targeted mutagenesis	Impaired	Meyer et al. 1996
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PAO25	IA130 (PAO25 <i>pvd-5</i>)	non-targeted mutagenesis	Impaired	Poole et al. 1996
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PA01	IAJ40M1 (PA01 <i>pvd-10</i>)	non-targeted mutagenesis	Impaired	Poole et al. 1996
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PA01	PA01Δ <i>fatC</i>	targeted mutation of <i>fatC</i> ^g	Impaired	Ochsner et al. 2002

^a *ppyR* encodes an uncharacterised membrane protein which, when deactivated, causes downregulation of 13 *pvd* genes (among others) and effective elimination of pyoverdine production (Attia et al. 2008b)

^b *pvdS* encodes an extracytoplasmic sigma-factor required for the production of pyoverdine, exotoxin and PrpL protease (Cunliffe et al. 1995; Miyazaki et al. 1995)

^c *pvdD* encodes a peptide synthetase essential for pyoverdine production (Merriman et al. 1995)

^d *pvdI* encodes a peptide synthetase essential for pyoverdine production (Lehoux et al. 2000)

^e *pvdA* encodes an ornithine hydroxylase and is essential for pyoverdine synthesis (Visca et al. 1994)

^f *pchD* is required for the synthesis of dihydroaeruginosate and pyochelin, a secondary siderophore (Serino et al. 1997)

^g *fatC* encodes a secretory apparatus required for at least 3 pyoverdine biosynthesis proteins and a ferripyoverdine receptor (Ochsner et al. 2002)

A related question is whether iron-limitation likely to be important given the specific type of infection we induced in these hosts? Pyoverdine production clearly benefits bacterial growth in mammalian hosts (Table 4.1) but, notably, even within a host it is not equally important across all tissue types, or over all time scales (acute vs. chronic), or for all infection routes (Table 4.1; Takase et al. 2000). *P. aeruginosa* does naturally associate with both insect larvae (Butcher 1981; Osborn et al. 2002) and lettuces (Kominos et al. 1972; Correa et al. 1991), but experimental infections in a laboratory can not capture all the features of natural, co-evolved infections. In nature, *P. aeruginosa* typically persists in or on these hosts at low densities and gives rise to few, if any, symptoms of disease. The relatively high inoculum dosages used in our experiments, the unnatural routes of infection and post-inoculation incubation conditions are all features designed to overwhelm host defenses and promote acute infection. It is conceivable that bacterial fitness differences resulting from different iron-metabolism profiles would become more pronounced in these host models in the context of more natural, chronic infections involving smaller pathogen loads.

With regard to (b), it is important to note that, while pyoverdine production is considered the most important of *P. aeruginosa*'s adaptations to iron limitation (Visca et al. 2007; Cornelis et al. 2008), alternative means of acquiring iron are also known. Cells can scavenge host-bound Fe(III) using the secondary siderophore, pyochelin (Cornelis and Matthijs 2002), or pyocyanin (Cox 1986), or can take up iron as haem or haemoglobin (Ochsner et al. 2000), or even as Fe(II) via a combination of redox molecules and ferrioxidasase (Newman and Kolter 2000; Huston et al. 2004). Indeed, *P. aeruginosa* appears to have evolved impressive flexibility with respect to iron acquisition, as evidenced by the many homologues of ferripyoverdine receptor genes in its genome (Cornelis and Matthijs 2002; De Chial et al. 2003; Poole and McKay 2003), the intraspecies diversity of "siderovars" (Cornelis et al. 1989; Meyer et al. 2002), the high genetic diversity and diversifying selection at the pyoverdine locus (Spencer et al. 2003; Smith et al. 2005), and the ability to use a variety of heterologous siderophores produced by other microbes (Guerinot 1994; Poole and McKay 2003). When presented in the laboratory with medium artificially depleted of iron, pyoverdine-defective strains show strongly

attenuated growth (Griffin et al. 2004; Ross-Gillespie et al. 2007), but it may be that, when growing in certain types of host tissues, these same mutant strains can use secondary pathways to access alternative iron sources (Takase et al. 2000). Indeed, there is still much to learn about how the *in vivo* physiology and metabolism of pathogens differs from that we see *in vitro* (Vasil and Ochsner 1999; Brown et al. 2008; Buckling and Brockhurst 2008).

Although we did not find growth differences across different infection types, we did, in some cases at least, observe attenuated virulence in infections involving pyoverdine-defective strains (Figure 4.2). This could potentially result because, aside from any growth benefits that may derive from enhanced iron metabolism, pyoverdine can contribute in a more direct way to the virulence of *P. aeruginosa*. The uptake of pyoverdine is known to positively regulate its own synthesis, as well as the production of two virulence factors, exotoxin A (ToxA) and an endoprotease (PrpL), which are major contributors to the ability of this bacterium to cause disease (Lamont et al. 2002; Beare et al. 2003). Thus, in wild type infections, the synthesis and uptake of pyoverdines could have enhanced damage to host tissue even though this did not translate to a significant growth advantage for the bacteria. That said, there is evidence to indicate that pyoverdine production can be facultatively regulated in response to iron limitation (Cunliffe et al. 1995; Leoni et al. 1996; Kim et al 2003; Kümmerli et al. *in press*). Under this scenario, if *in vivo* conditions were not iron-limited, as we hypothesise, pyoverdine production by wild type cells should have been deactivated (or low). This would account for the lack of growth differences between treatments, but does not explain the differences in virulence.

Siderophores can be shared extracellularly as public goods, and where this occurs we would expect that pyoverdine-defective cheats could invade a population of pyoverdine-producers and increase in relative frequency. However, our waxmoth infection data indicate that cheats had lower relative fitness than cooperators in co-infection, as found by Harrison et al. (2006). Does this suggest then that social cheats have no place in an infection context? On the contrary, the relative costs and benefits of cooperation vs. cheating are highly context dependent, and are expected to vary

as, for example, the environment becomes more benign (Griffin et al. 2004), or as the population structure changes (Griffin et al. 2004; Harrison et al. 2006; Ross-Gillespie et al. 2007). Indeed, in longitudinal studies of *P. aeruginosa* infections in the cystic fibrosis lung, average pyoverdine production per cell is seen to decrease over the course of the infection, while concomitantly, pyoverdine-defective mutants increase in frequency (De Vos et al. 2001; Smith et al. 2006). Whether this reflects ongoing social conflict, or merely a decreased need for siderophores as more iron becomes available in the CF lung (Stites et al. 1999), remains to be resolved.

5. Social conflict over quorum sensing and its consequences for bacterial pathogenesis

5.1. Summary

Bacteria coordinate and regulate much of their activity using ‘quorum sensing’ (QS) chemical signaling but, as *in vitro* experiments demonstrate, this is cooperative behaviour, involving costs to the individual and benefits at a group level. As such, QS is vulnerable to exploitation by QS-deficient cheats. This exploitation can have significant fitness consequences at both the strain and population level, which, in the context of co-infecting pathogens, could potentially impact on the virulence experienced by a host. Here we use the bacterium *Pseudomonas aeruginosa* to test whether QS functions as a social trait *in vivo* in experimental infections in insects (*Galleria mellonella*) and plants (*Lactuca sativa*). Our data indicate that QS does not provide a bacterial growth advantage in these infection models and accordingly, we found no consistent evidence of social exploitation among strains. Finally, although virulence did vary across infection types, it was not attenuated in co-infections of wild type and QS-deficient mutants. Thus, our data do not support the idea that QS functions as a cooperative trait *in vivo* in these host models. More generally, we survey evidence from experimental infection studies for the effect of QS-disruption on virulence. We conclude that, while QS frequently plays an important role in virulence, notable exceptions exist for some host taxa and certain infection types and these limitations should be recognized in the design of QS-targeted therapies.

5.2. Introduction

Microbiology has been revolutionised in recent decades by the discovery that bacteria possess elaborate chemical signaling systems by which they can gain information about other cells in their local environment and coordinate their behaviour accordingly (Fuqua et al. 2001; Bassler and Losick 2006; Camilli and Bassler 2006; Williams et al. 2007). These so-called “quorum sensing” (QS) systems operate by the extracellular release, dissemination and uptake of small molecules which, typically once they attain a threshold concentration (i.e. quorum),

subsequently trigger the expression or repression of target genes. QS systems have been shown to operate in a wide variety of bacterial taxa and make it possible for bacteria to act cooperatively in accessing nutrients, killing competitors, defending themselves against biotic or abiotic threats, migrating to more favourable habitats, or banding together into multicellular aggregations better suited to the rigours of their environment (e.g. biofilms, fruiting bodies; Williams et al. 2007).

One of the most intensively-studied and best-characterized QS systems is that of *Pseudomonas aeruginosa*, a Gram-negative bacterium found in most terrestrial and aquatic environments and an opportunistic pathogen of a wide range eukaryotes, from amoebae to mammals (D'Argenio 2004; Juhas et al. 2005; Venturi 2006; Case et al. 2008). In humans, *P. aeruginosa* is a notorious cause of infections among burned, intubated, post-operative and immune-compromised patients and is a particular problem in the lungs of cystic fibrosis sufferers, where it establishes chronic, stubborn and ultimately fatal infections (Cross et al. 1983; Govan and Deretic 1996; Lyczak et al. 2000; Lyczak et al. 2002; Harrison 2007). The QS regulatory apparatus in this species comprises two subcircuits, Las and Rhl, which are mediated by the N-acyl homoserine lactone signal molecules 3-oxo-C12-HSL and C4-HSL, respectively. In each case there is a synthase gene (*lasI* or *rhlI*) which encodes the signal molecule and a second gene (*LasR* or *rhlR*) encoding a cognate receptor which both regulates target genes and amplifies the signal by autoinducing further signal production (Gambello et al. 1993; Latifi et al. 1995). The organization of the Las and Rhl systems is not yet fully understood, although Las is thought to be hierarchically dominant because it positively regulates the Rhl system (Latifi et al. 1996; Pesci et al. 1997; McKnight et al. 2000; de Kievit et al. 2002; Schuster and Greenberg 2006; Venturi 2006), via interaction with a third system involving a 2-alkyl-4-quinolone signal (PQS; Diggle et al. 2003; Wade et al. 2005; Dubern and Diggle 2008).

Quorum sensing is considered to play a vital role in the basic biology and pathogenesis of *P. aeruginosa*. Indeed, transcriptome studies suggest that over 300 genes – between 6 and 10% of the genome – could be under QS control (Hentzer et

al. 2003; Schuster et al. 2003; Wagner et al. 2003). In particular, the Las and Rhl systems are known to regulate the expression of multiple extracellular virulence factors including elastase, alkaline protease, exoenzyme S, exotoxin A, rhamnolipids, pyocyanin, hydrogen cyanide, neuraminidase, haemolysin, lectins, catalase and superoxide dismutases (see reviews by Passador et al. 1993; Brint and Ohman 1995; Van Delden and Iglewski 1998; Juhas et al. 2005). QS is involved in iron acquisition (Stintzi et al. 1998; Hentzer et al. 2003; Ren et al. 2005), it is required for swarming (Köhler et al. 2000; Overhage et al. 2008) and twitching motility (Glessner et al. 1999; although see also Beatson et al. 2002), it has an anticompétitor function (Kaufmann et al. 2005; Qazi et al. 2006; McAlester et al. 2008), it positively regulates the injection of virulence factors into host cells via type III secretion (Hogardt et al. 2004; Bleves et al. 2005) and it may also, under certain conditions at least, play a role in the formation and maintenance of biofilms (Kjelleberg and Molin 2002; Parsek and Greenberg 2005; Kirisits and Parsek 2006; Patriquin et al. 2008). QS systems have been shown to operate in infected tissues, especially the cystic fibrosis lung (Stickler et al. 1998; Storey et al. 1998; Geisenberger et al. 2000; Singh et al. 2000; Wu et al. 2000; Erickson et al. 2002; Middleton et al. 2002; Ward et al. 2003; Wu et al. 2003) and experimental studies with model hosts (including rodents, plants, insects, nematodes and amoebae) have demonstrated that mutations in *las* or *rhl* genes typically reduce the capacity of *P. aeruginosa* to establish and maintain infections (Table 5.1; see also reviews by Rumbaugh et al. 2000; Donabedian 2003; Smith and Iglewski 2003).

Aside from its evident importance to *P. aeruginosa*, QS is fundamentally a social trait with complex fitness consequences for both signalers and receivers, and as such, it can lead to conflicts of interest (Brookfield 1998; Brown and Johnstone 2001; Keller and Surette 2006; West et al. 2006; Diggle et al. 2007a; Diggle et al. 2007b; Hense et al. 2007). A large body of evolutionary theory predicts that communication and cooperation are vulnerable to exploitation (Hamilton 1964; Frank 1998; Maynard Smith and Harper 2003; Lehmann and Keller 2006). For instance, In the case of QS, there could be cheats that either (a) do not produce signal but still benefit by monitoring the signals of others ('signal-negative'), or (b) do not respond to

others' solicitations ('signal-blind') and thus avoid the costs of exoproduct production, etc. while still benefiting from the QS-controlled contributions of others (Haas 2006). Meanwhile, signal-blind cheats could still produce (or even overproduce) signal to 'coerce' other cells into greater levels of cooperation (Brown and Johnstone 2001; Keller and Surette 2006).

QS mutants, especially signal blind *lasR*-defective mutants, have been found in *P. aeruginosa* populations from diverse sources and are known to spontaneously arise and spread in artificial cultures and natural infections alike (Hamood et al. 1996; Cabrol et al. 2003; Denervaud et al. 2004; Schaber et al. 2004; Zhu et al. 2004; Heurlier et al. 2005; Lee et al. 2005b; Salunkhe et al. 2005; Smith et al. 2006; D'Argenio et al. 2007; Fothergill et al. 2007; Lujan et al. 2007; Sandoz et al. 2007; Bosgelmez-Tinaz and Ulusoy 2008). While this pattern could arise if the loss of QS is simply an adaptation to the host environment, an alternative explanation is that these mutants are social cheats which spread at the expense of cooperators (West et al. 2006; Sandoz et al. 2007). Concordant with this idea, Diggle et al. (2007b) demonstrated in a series of elegant experiments that both *lasI* and *lasR* mutants can function as cheats *in vitro*. More recently, we showed that these patterns hold *in vivo* too (Rumbaugh et al, submitted). Using a mouse model, we showed that under co-infection conditions, QS-mutants could exploit cooperators (wild-type cells) and consequently increase in frequency, and that this social conflict reduced the virulence of such infections relative to those involving only wild-type QS-proficient bacteria.

In this study, we explore the generality of these results. We consider two non-mammalian host models, an insect (*Galleria mellonella*) and a plant (*Sativa lactuca*), which we experimentally infect with QS-defective *P. aeruginosa* strains derived from the widely studied PA01 background (Stover et al. 2000). Specifically, we test the predictions that (a) QS enhances *P. aeruginosa* growth in an infection context and (b) is, in turn, detrimental to the host; (c) QS-defective mutants 'cheat' by exploiting the QS-related cooperative behaviour of co-infecting wild type cells *in vivo* and (d) such social conflict can attenuate both the bacterial productivity and (e) the virulence of such co-infections relative to wild type infections.

5.3. Methods

Bacterial strains and model hosts

We considered two types of *Pseudomonas aeruginosa* QS mutants, defective for different parts of the LasI/LasR signaling system. We derived “signal-negative” and “signal-blind” cheats in a PA01 ATCC15692 background by targeted disruption of the genes *lasI* and *lasR* respectively. Briefly, we used standard conjugation techniques to transfer suicide vector plasmids from *E. coli* S17-1 donor strains to our PA01 wild type, where the latter was first sensitized to mating by overnight growth at 42°C. Specifically, the conjugations involved plasmids pSB219.8A and pSB219.9A (carrying *lasI*::Gm and *lasR*::Gm respectively; Beatson et al. 2002). We isolated single-crossover conjugants from Pseudomonas Isolation Agar (Fluka 17208) supplemented with 100 µg/ml gentamycin, and subsequently grew them on 5% sucrose to select against retention of the plasmid (which promotes host cell sensitivity to sucrose via the genes *sacBR*). Thus, we generated stable double-crossover QS mutants, in which there is loss-of-function disruption of a single gene (either *lasI* or *lasR*) but no cost of plasmid maintenance.

To allow us to discriminate between colonies of cooperator vs. cheat strains, we generated duplicate versions of our wild-type and mutant strains tagged with bioluminescence genes from the proteobacterium *Photobacterium luminescens*. Using similar standard protocols to those above, we conjugated the promoter-less *luxCDABE* cassette (via the 12538 bp mini-CTX-*lux* plasmid; Becher and Schweizer 2000) from an *E. coli* S17-1 λ pir donor into the chromosome of our target *P. aeruginosa* strains. The resulting stable single-crossovers, isolated from agar supplemented with 200 µg/ml tetracycline, constitutively express a low level of bioluminescence which is clearly detectable in long-exposure images in an otherwise dark environment. We detected no significant growth differences *in vitro* between tagged and untagged versions of our strains (data not shown), suggesting that the metabolic costs of *lux* expression are negligible under standard growth conditions.

We performed infections with both an insect and a plant host. For the insect host, we used larvae of the greater waxmoth (*Galleria mellonella*; Livefood UK; <http://www.livefoods.co.uk>). As final instar larvae, waxmoths did not feed, and under refrigeration maintained good body condition for up to a week following purchase. Our methodology is derived from that of previous studies (Lysenko 1963; Jander et al. 2000; Harrison et al. 2006). For the plant host, we obtained commercially-grown Romaine lettuces (*Lactuca sativa*) from a local supermarket, and infected them within two hours of purchase. This lettuce infection model, on which we based our methodology, has been widely used to study *P. aeruginosa* pathogenesis (e.g. Rahme et al. 1997; Filiatrault et al. 2006; Vives-Florez and Garnica 2006; Wagner et al. 2007).

Preparation of bacterial inocula

We conducted infection experiments one strain pair at a time, with at least two blocks per pair. For each pair, we grew separate overnight cultures of the wild type and a derived QS mutant from freezer stock, using 6 ml of standard Kings B medium in an orbital shaker (37°C, 200 r.p.m.). After 24 hours, we prepared our treatment inocula. First, we twice pelleted cells by centrifugation (3 min; 6000 r.p.m.) and washed them in buffer (10 mM MgSO₄ in the case of lettuce infections; 0.8% NaCl solution in the case of waxmoth infections). We set up three treatments: pure cooperator ('+'); pure cheat ('-'); a mix of + and - ('M'; at a volumetric ratio of 1:1 in the case of the waxmoth model or 3:1 in the case of lettuce model) as well as a control treatment, ('0'; buffer only, as above). For the waxmoth model, we used untagged PA01 wild-type (cooperator) and lux-tagged QS mutants (cheats), at an infecting dosage of $3-5 \times 10^3$ CFU. For lettuce infection assays we used a lux-tagged PA01 wild-type (cooperator) vs. untagged QS mutants (cheats), at a dose of $\sim 1 \times 10^7$ CFU. In all cases, we administered doses using a 10 µl precision syringe (<http://www.hamiltoncompany.com>) fitted with a 26S gauge needle, which was sterilised between each treatment using 100% EtOH and a sterile water rinse.

(a) Waxmoth model

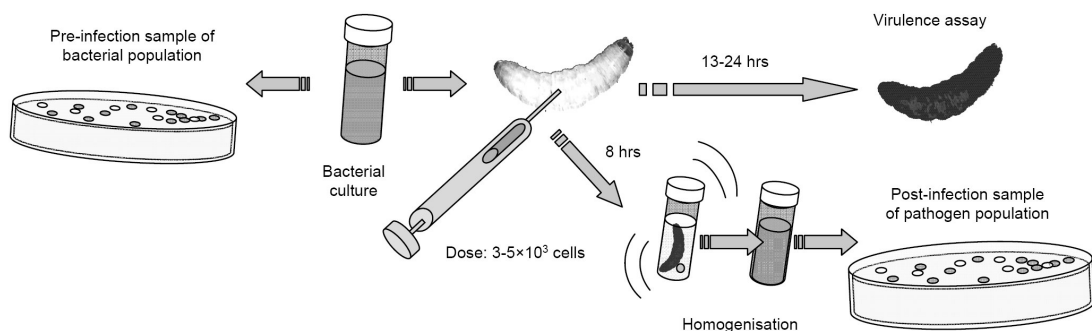
We conducted separate assays for virulence and bacterial growth, although in either case we performed the initial infections in the same way. In the case of virulence assays, for each strain pair in turn, we randomly allocated replicates to treatments split equally across two blocks. For bacterial growth assays, meanwhile, we obtained data from one single-block experiment per strain pair. For each larva, we weighed it to the nearest 0.1 mg, swabbed it with 70% ethanol to kill surface contaminants, and injected it in the abdomen between the first prolegs. Following injection, we randomly distributed larvae to individual wells of 24-well sterile microtitre plates and incubated them at 37°C. Finally, we took samples of the inocula, plated them to LB agar and, following overnight incubation at 37°C, counted CFUs to obtain estimates of initial cell densities. For mixed infections, we also photographed plates in a sealed cabinet (Fluorchem SP Light Cabinet, operated with AlphaPart 11 Ease software; Alpha Innotech <http://www.alphainnotech.com>) then digitally overlaid false-coloured screens of regular (illuminated) and long-exposure (6 min; non-illuminated) photographs to yield a composite image in which *lux*-tagged and untagged CFUs could easily be distinguished. From these photographs, we obtained an estimate of the starting ratio of cooperators to cheats.

For virulence assays, we monitored mortality at hourly intervals between 13 and 24 hours post-inoculation. We recorded larvae as dead if they failed to respond to mechanical stimulation of the head and prolegs. Distinct melanism was also evident in dead larvae (Figure 5.1A). Control treatment larvae showed negligible mortality.

For bacterial growth assays, we retrieved larvae after 8 hr incubation, and homogenized them individually in 0.5 ml 0.8% NaCl by rapid shaking with a sterile ceramic bead (FastPrep FP120; <http://www.mpbio.com>; 4 m/s for 2 min). We centrifuged the homogenates at low speed (1000 r.p.m.; 3 min) to separate host tissues into distinct bands, then took samples from the liquid (haemolymph) fractions, and after appropriate serial dilution in 0.8% NaCl, spread them using

automated spiral plating (EddyJet; <http://www.iul-inst.com>) to plates of LB agar supplemented with 15 µg/ml ampicillin to restrict the growth of non-target bacteria (this concentration does not impede growth in *Pseudomonas aeruginosa*). We then incubated these plates overnight at 37°C then counted CFUs to obtain estimates of the endpoint cell densities achieved by the infecting populations. As before, we produced composite photographs of the plates to in which lux-tagged CFUs could be easily distinguished from untagged CFUs and from these images we estimated the endpoint ratios of cooperators to cheats. When estimating the final pathogen population density within a given larva, we had to consider how size differences among larvae could affect the total volume of homogenate from which we drew our sample. In a separate experiment (data not shown), we regressed larval mass against the volume of liquid larvae displaced, thereby establishing that larval volume (ml) = $1.032 \times \text{larval mass (g)}$. We applied this correction factor to each homogenized host such that our estimates of final pathogen density were for the complete host (i.e. the entire infecting population), rather than per some unit of host tissue.

(A) WAXMOTH INFECTION MODEL



(B) LETTUCE INFECTION MODEL

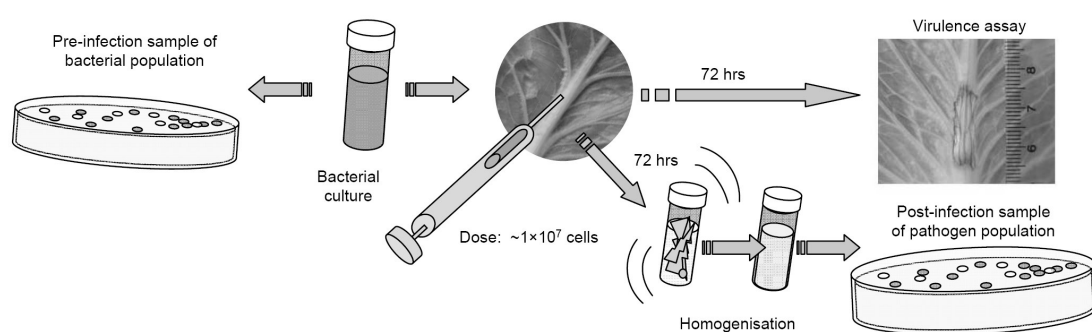


Figure 5.1. Experimental protocols used in (A) waxmoth and (B) lettuce infection models.

(b) Lettuce model

From each head of lettuce, we selected four leaves of similar size (one per treatment), which we washed briefly with 0.1% bleach to kill surface contaminants and then patted dry with sterile tissue. We cut 50 mm diameter discs from the centre of the leaves using a cookie cutter, and placed them individually in 10 cm petrie dishes. We then inoculated leaf discs with bacteria (see dosage details above) by injection into the midrib using a 10 µl RN Hamilton syringe fitted with a 26S needle. Finally, we placed the leaf discs, in their individual petrie dishes, in a sealed container kept humid by the addition of filter papers moistened with 10 mM MgSO₄ and incubated them at room temperature on the laboratory bench. Samples of the inocula were diluted and plated to LB agar. Following overnight incubation at 37°C, we counted CFUs to obtain estimates of the starting cell densities. We also photographed mixed inocula plates as described above in order to estimate starting ratios of wild type to mutant CFUs.

After 72 hours incubation, we assayed virulence by measuring the maximum extent of tissue damage along the midrib of the leaf disc, taking our measurements post-hoc from scale-standardized digital photographs (Figure 5.1B). Control leaf discs, injected with MgSO₄ buffer only, showed negligible tissue damage. We then suspended whole leaf discs in 2 ml 10 mM MgSO₄ and homogenized them by rapid shaking with a sterile ceramic bead (FastPrep FP120; 4 m/s for 2 min). We took samples of the homogenate and, after serial dilution, spread them using automated spiral plating to LB agar supplemented with 15 µg/ml ampicillin. We incubated these plates overnight at 37°C then counted CFUs to obtain estimates of the endpoint cell densities achieved by the infecting populations and, for mixed infections, we took photographs (as per above) from which we estimated endpoint ratios of cooperators to cheats.

Statistical analyses

We calculated bacterial growth for each host, defined as the number of doublings an infecting population underwent during the observed period of infection (Lenski et al.

1991). In the case of mixed infections (treatment ‘M’), we were also able to estimate, separately, the growth of the cooperator and cheat subpopulations, since we had estimates for total population size and proportion of each strain type at both the start and end points of the infection period. We analysed bacterial growth data using linear mixed effects models or, where appropriate, ANOVA, with larval mass included as a covariate. For each mixed infection, we calculated values for the relative fitness of cheats (v), by comparing the frequency of cheats at the beginning and end of each infection. Specifically, v is given by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheats in the inoculum and x_2 is their final proportion (Ross-Gillespie *et al.* 2007; see also “competitive index”; Beuzon and Holden 2001). The value of v therefore signifies whether cheats increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We tested whether v differed significantly from 1 using a Z-test. We analysed waxmoth mortality data using parametric survival analysis, specifying the commonly used Weibull error structure, which allows the probability of death to vary over time (Carroll 2003). The maximal model included a blocking factor but, since it had no significant effect, it was removed from the final model. Since lettuce infections involved multiple replicate blocks per strain pair (with treatments equally represented in each), we began our analyses with maximal models including the random effects *block* and, nested within it, *lettuce identity*. Where these random effects comprised a substantial portion of the total error variance (>5%) they were retained in the model, but in other cases, they were excluded and the resulting minimal model was then a 1-way ANOVA. All analyses were performed in S-Plus 8.0 (<http://www.insightful.com>).

5.4. Results

(a) Waxmoth model

Data on bacterial growth within waxmoth hosts are summarized in Figure 5.1A. For the strain pair featuring the signal-blind mutant, PA01 Δ *lasR*, there was an *in vivo* growth difference across treatments ($F_{2,26} = 4.434$, $p = 0.022$), in that mixed infections showed more growth than the mutant alone (post-hoc Tukey test showed

95% confidence intervals excluded 0 in the 'M' vs. '-' comparison only), whereas for the strain pair with the signal-negative mutant, PA01 Δ *lasI*, we found no significant growth differences across treatments ($F_{2,26} = 1.932$, $p = 0.165$). Next, we compared growth of the QS-defective mutants ('cheats') only, in single infection versus mixed infection. Signal-blind mutants did show a growth advantage in co-infection versus single infection (ANOVA: $F_{1,18} = 10.265$, $p = 0.005$) while for signal-negative mutants, the opposite patterns emerged – growth was relatively higher in single infection (ANOVA: $F_{1,19} = 7.429$, $p = 0.013$). Finally, we tested whether mutants had a relative fitness advantage over the wild type within a co-infection. Signal blind mutants were relatively fitter than the wild type in co-infections ($v = 1.28$; 1-tailed Z-test of $v > 1$; $t = 2.63$, d.f. = 9, $p = 0.014$), whereas signal-negative mutants were relatively less fit than the wild type ($v = 0.46$; 1-tailed Z-test of $v < 1$; $t = 15.84$, d.f. = 10, $p < 0.001$). Virulence varied significantly across different infection treatments (Figure 5.3A; parametric survival analyses; signal-blind mutant: $X^2 = 13.15$, d.f. = 2, $n = 89$, $p < 0.002$; signal-negative mutant: $X^2 = 21.59$, d.f. = 2, $n = 90$, $p < 0.001$) but not in a pattern generally concordant with our growth data reported above (Figure 5.2A). Single infections with the signal-blind mutant were not significantly different in virulence to infections with the wild type ($z = 1.47$, $p = 0.143$); whereas in the case of the signal-negative mutant, the mutant was significantly more virulent than the wild type ($z = -2.07$, $p = 0.039$). In either case, co-infections involving a mixture of PA01 wild type and a QS-defective mutant resulted in significantly greater virulence (i.e. quicker waxmoth death) than did single infections of the wild type strain (signal-blind mutant: $z = -2.26$, $p = 0.024$; signal-defective mutant: $z = -5.12$, $p < 0.001$).

(b) Lettuce model

Total bacterial growth did not differ significantly across the three infection treatments (Figure 5.2B; LMER: $F_{2,36} = 0.091$, $p = 0.913$). Considering the QS-mutant strain only, there was no evidence for a growth advantage in co-infection relative to single infection (Figure 5.2B; ANOVA: $F_{1,25} = 0.189$; $p = 0.668$), although within a co-infection, this strain did show relative fitness greater than 1 (mean 2.297; 1-tailed Z-test: $t = 3.047$, d.f. = 11, $p = 0.006$), suggesting that it was able to increase its proportional representation in a mixed population at the expense of the wild type

strain. In contrast to the patterns we observed in waxmoths, our *a priori* predictions regarding virulence were well met in the lettuce infection model (Figure 5.3B). These differences in virulence did not, however, correspond to differences in growth reported above. The QS-defective mutant strain PA01 Δ *lasR* resulted in significantly less tissue damage than infections with the PA01 wild type, while co-infections involving a mixture of the two strains had intermediate virulence (LMER: $F_{2,94} = 12.2935$, $p < 0.0001$).

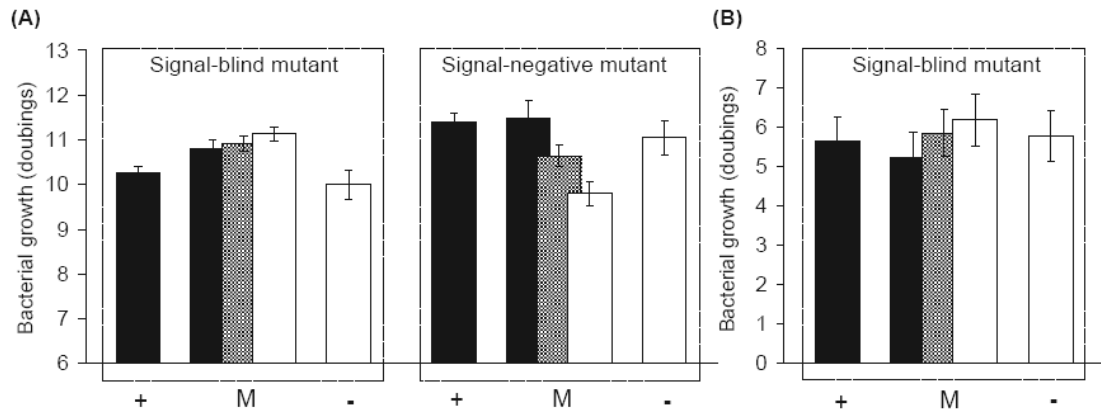


Figure 5.2. Variation in bacterial growth across different strains and infection types for (A) waxmoth and (B) lettuce infection models. Black bars refer to populations of cooperator cells (wild type PA01), either growing alone (+) or in a co-infection (M). White bars refer to populations of cheat cells (signal-blind, PA01 Δ *LasR* or signal-negative, PA01 Δ *LasI*, respectively), either growing alone (-) or in a co-infection (M). Checked bars refer mixed populations of cooperators and cheats. Error bars indicate one standard error.

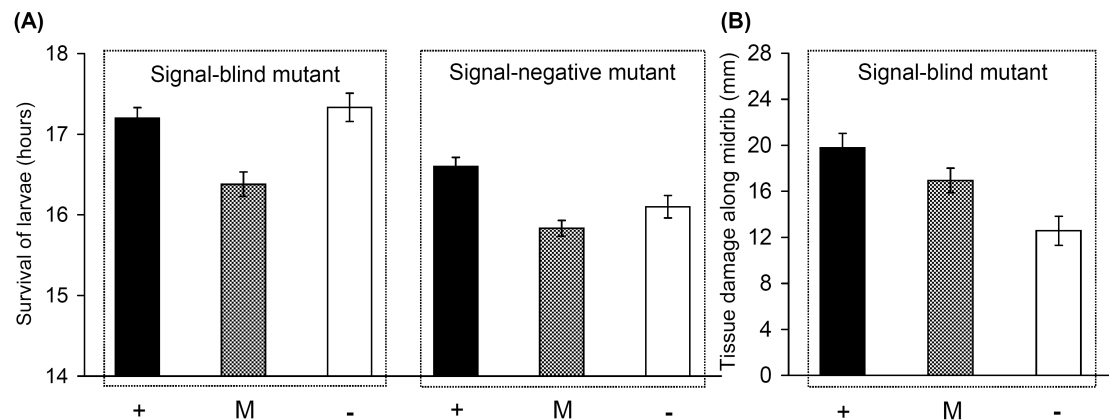


Figure 5.3. Variation in virulence across different strain pairs and infection types for (A) waxmoth and (B) lettuce infection models. Black bars indicate infections with the cooperator strain (wild type PA01) only. White bars indicate infections with cheat strains only (signal-blind, PA01 Δ *LasR* or signal-negative, PA01 Δ *LasI*, respectively). Checked bars indicate co-infections with both cooperators and cheats (M). Error bars indicate one standard error.

5.6. Discussion

In this study we tested the hypothesis that, given (a) QS promotes *P. aeruginosa* growth in an infection context and (b) is, in turn, detrimental to the host; (c) QS-defective mutants can ‘cheat’ by exploiting the QS-related cooperative behaviour of co-infecting wild type cells *in vivo* and (d) such social conflict can attenuate both the bacterial productivity and (e) the virulence of such co-infections relative to wild type infections. These various predictions are summarized in table 5.1.

Table 5.1. Summary of results from experiments to test whether QS functions as a social trait in an infection context and what the implications of this are for virulence.

Prediction	Support found?		
	Waxmoth model		Lettuce model
	LasR	lasI	lasR
(a) QS-proficient strains show more growth <i>in vivo</i> than QS deficient strains	✗	✗	✗
(b) Infections with QS-proficient strains are more detrimental to their host than those involving QS-deficient strains	✗	✗	✓
(c) QS-defective cheats achieve greater relative fitness <i>in vivo</i> than QS-proficient cells	✓	✗	✓
(d) Mixed populations of QS-proficient and –deficient strains have relatively attenuated productivity <i>in vivo</i>	✗	✓	✗
(e) Coinfection with QS-proficient and –deficient strains leads to attenuated virulence	✗	✗	✓

Firstly, and crucially, we found no support for (a). Considering single infections only (Figure 5.2), we see that disruption of QS had a negligible effect on the growth of bacteria *in vivo* in either infection model (Figure 5.2). The generally consistent levels of growth did not, however, translate to consistent virulence. Instead, for (b) we found evidence of, respectively, (i) a QS-mediated enhancement of virulence (Figure 5.3A, signal-negative mutant), (ii) no difference (Figure 5.3A, signal-blind mutant) or (iii) QS-mediated attenuation of virulence (Figure 5.3B). This apparent disassociation between growth and virulence is interesting and we return to it later in this section. Considering (c), that QS mutants can act as cheats in co-infections, we found that signal-blind mutants could indeed have high relative fitness in such conditions (Figure 5.2) but signal-negative mutants did not. As for (d) and (e), we found that co-infections with wild type and QS mutants could yield either enhanced

(Figure 5.2A; signal-blind), attenuated (Figure 5.2A signal-negative) or equivalent growth (Figure 5.2B) relative to a wild type infection; and either greater (Figure 5.3A) or reduced (Figure 5.3B) virulence, depending on the type of mutant and the model host involved. Taken together, our results do not support the hypothesis that QS is an important and exploitable cooperative trait in these infection models (West and Buckling 2003).

We found several instances where bacterial growth and virulence were disassociated (Figure 5.2; Figure 5.3) which suggests that the expression of QS may negatively affect host fitness without having significant consequences for bacterial fitness (at least over the short term). This has been reported in other models (Cowell et al. 1998; Silo-Suh et al. 2002; Lesprit et al. 2003; Stoltz et al. 2008) and is interesting in light of the fact that QS has been touted as a potential target for novel anti-infective drugs and it is now recognized that for any such therapy to avoid inducing pathogen counter-adaptation, it must reduce virulence without affecting cell viability (Bjarnsholt and Givskov 2007; Lesic et al. 2007).

How do our results compare with previous work? To our knowledge, we and Rumbaugh et al (submitted) are the first to investigate the *in vivo* social dynamics and virulence consequences of co-infections between Las-mutants and wild type *P. aeruginosa* but several other groups have performed single-strain infections with various model hosts to investigate the role of QS (i.e. hypotheses a and b; Table 5.2). Notably, Jander et al. (2000), using a wax moth model similar to ours, report clearly reduced virulence for a PA14-derived *lasR* mutant while Wagner et al. (2007) found attenuated virulence in PA01 QS mutants (including $\Delta lasR$) in a lettuce model. Neither study, however, reported the bacterial growth trends underlying this pattern.

It is often suggested in the literature that disruption of QS attenuates pathogenesis (Rumbaugh et al. 2000; Donabedian 2003; Smith and Iglewski 2003) but mounting evidence suggests this is not always the case (Table 5.2) and it is becoming increasingly clear that to properly understand the role of QS in bacterial pathogenesis, strain-and host-specific details must be taken into consideration

Table 5.2. [part I] Evidence for the effects of las/rhl quorum sensing disruption on the virulence of experimental infections

Host organism	Infection model	Control strain	Gene(s) disrupted	Strain identity	Mutation type	Strain reference	Virulence of QS-defective strain relative to control	Reference
PROKARYOTES								
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PT5	lasI	PT466	targeted mutation	26	Impaired	10
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PA14	lasR	12A1	targeted mutation	54	Impaired	43
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PA14	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	32	No difference	43
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PT5	lasR	PT498	targeted mutation	26	Impaired	10
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PA01	lasR	?	?	?	Impaired	10
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PA01	lasR	22D10	clinical isolate (intubated patient)	13	No difference	1
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PT5	rhlI	PT454	targeted mutation	26	Impaired	10
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PT5	rhlI	PT462	targeted mutation	26	Impaired	10
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PT5	lasI/rhlI	PT502	targeted mutation	10	Impaired	10
<i>Dictyostelium discoideum</i>	Feeding (growth inhibition)	PT5	lasR/rhlR	PT531	targeted mutation	26	Impaired	10
<i>Rhynchomonas nasuta</i>	Feeding (growth inhibition)	PA01	lasR	PAO-R1	targeted mutation	21	No difference	35
<i>Rhynchomonas nasuta</i>	Feeding (growth inhibition)	PA01	lasR/rhlR	PAO1ΔlasR ΔrhlR	targeted mutation	3	No difference	35
PLANTS								
<i>Arabidopsis thaliana</i>	Injured root tips	PA01	lasI	PAO214	targeted mutation	22	No difference	58
<i>Arabidopsis thaliana</i>	Leaf infiltration	PA14	lasR	12A1	targeted mutation	54	Impaired	54
<i>Arabidopsis thaliana</i>	Leaf infiltration	PA14	lasR	PA14ΔlasR	targeted mutation	45	No difference	44
<i>Arabidopsis thaliana</i>	Injured root tips	PA01	rhlI	PAO210	targeted mutation	22	No difference	58
<i>Arabidopsis thaliana</i>	Injured root tips	PA01	lasI/rhlI	PAO216	targeted mutation	22	No difference	58
<i>Lactuca sativa</i>	Leaf infiltration	PA01	lasI	PAO-JP1	targeted mutation	40	Impaired	57
<i>Lactuca sativa</i>	Leaf infiltration	PA01	lasR	PAO-R1	targeted mutation	21	Impaired	57
<i>Lactuca sativa</i>	Leaf infiltration	PA01	rhlI	PDO100	targeted mutation	5	Impaired	57
<i>Lactuca sativa</i>	Leaf infiltration	PA01	rhlR	PDO111	targeted mutation	5	Impaired	57
<i>Lactuca sativa</i>	Leaf infiltration	PA01	lasI/rhlI	PAO-JP2	targeted mutation	40	Impaired	57
<i>Lactuca sativa</i>	Leaf infiltration	PA01	lasR/rhlR	PAO-JP3	targeted mutation	40	Impaired	57
<i>Medicago sativa</i>	Injured root tips	FRD1	lasR	FRD-SS905	targeted mutation	50	No difference	50
<i>Medicago sativa</i>	Injured root tips	FRD1	rhlR	FRD-SS1206	targeted mutation	50	Impaired	50
<i>Ocimum basilicum</i>	Injured root tips	PA01	lasI	PAO214	targeted mutation	22	No difference	58
<i>Ocimum basilicum</i>	Injured root tips	PA01	rhlI	PAO210	targeted mutation	22	Impaired	58
<i>Ocimum basilicum</i>	Injured root tips	PA01	lasI/rhlI	PAO216	targeted mutation	22	Impaired	58
<i>Populus trichocarpa</i>	Root colonization	PA01	rhlI	PA3476-UBC	targeted mutation	30	No difference	2
<i>Hordeum vulgare</i>	Seed germination inhibition	PA01	rhlI	PA3476-UBC	targeted mutation	30	Enhanced	2

Table 5.2. [part II] Evidence for the effects of las/rhl quorum sensing disruption on the virulence of experimental infections

Host organism	Infection model	Control strain	Gene(s) disrupted	Strain identity	Mutation type	Strain reference	Virulence of QS-defective strain relative to control	Reference
INSECTS								
<i>Drosophila melanogaster</i>	Thoracic puncture	PA01	lasI	?	targeted mutation	?	Impaired	53
<i>Drosophila melanogaster</i>	Adult feeding	PA01	lasR	LES400	clinical isolate (cystic fibrosis lung)	39	Enhanced	48
<i>Drosophila melanogaster</i>	Adult feeding	PA01	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	32	Impaired	33
<i>Drosophila melanogaster</i>	Thoracic puncture	PA01	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	32	Impaired	33
<i>Drosophila melanogaster</i>	Thoracic puncture	PA14	lasR	PA14ΔlasR	targeted mutation	44	Impaired	44
<i>Drosophila melanogaster</i>	Thoracic puncture	PA01	lasI/rhlI	?	targeted mutation	?	Impaired	53
<i>Drosophila melanogaster</i>	Thoracic puncture	PA01	lasI/rhlI	?	targeted mutation	?	No difference	53
<i>Drosophila melanogaster</i>	Thoracic puncture	PA14	lasI/lasR	PA15	clinical isolate (urinary tract infection)	31	Impaired	28
<i>Drosophila melanogaster</i>	Adult feeding	PA01	lasR/rhlR	6106	clinical isolate (cystic fibrosis lung)	16	No difference	17
<i>Drosophila melanogaster</i>	Adult feeding	PA01	lasR/rhlR	6106	clinical isolate (cystic fibrosis lung)	16	Impaired	33
<i>Drosophila melanogaster</i>	Thoracic puncture	PA01	lasR/rhlR	?	targeted mutation	?	Impaired	53
<i>Drosophila melanogaster</i>	Thoracic puncture	PA01	lasR/rhlR	6106	clinical isolate (cystic fibrosis lung)	16	Impaired	33
<i>Galleria mellonella</i>	Abdominal injection	PA01	lasI	PA01ΔlasI	targeted mutation	56	Enhanced	56
<i>Galleria mellonella</i>	Abdominal injection	PA14	lasR	12A1	targeted mutation	54	Impaired	24
<i>Galleria mellonella</i>	Abdominal injection	PA01	lasR	PA01ΔlasR	targeted mutation	56	No difference	56
<i>Galleria mellonella</i>	Abdominal injection	PA01	lasI/rhlI	P147	targeted mutation	15	No difference	44
NEMATODES								
<i>Caenorhabditis elegans</i>	Feeding (paralysis)	CDP46	lasR	CDP11	targeted mutation	12	Impaired	12
<i>Caenorhabditis elegans</i>	Feeding (paralysis)	PA01	lasR	PAO-R1	targeted mutation	21	Impaired	20
<i>Caenorhabditis elegans</i>	Feeding (slow-killing)	PA14	lasR	12A1	targeted mutation	54	Impaired	51
<i>Caenorhabditis elegans</i>	Suspension (feeding inhibition)	PA01	lasR	PAOR	targeted mutation	27	Impaired	51
<i>Caenorhabditis elegans</i>	Feeding (paralysis)	CDP30	rhlR	CDP32	targeted mutation	12	Impaired	12
<i>Panagrellus redivivus</i>	Feeding (killing)	PA01	lasI	PAO1ΔlasI	targeted mutation	3	Impaired	6
<i>Panagrellus redivivus</i>	Feeding (killing)	PA01	lasI/rhlI	PAO1ΔlasI ΔrhlI	targeted mutation	3	Impaired	6
<i>Panagrellus redivivus</i>	Feeding (killing)	PA01	lasI/lasR	PAO1ΔlasRI	targeted mutation	3	Impaired	6
<i>Panagrellus redivivus</i>	Feeding (killing)	PA01	lasR/rhlR	PAO1ΔlasR ΔrhlR	targeted mutation	3	Impaired	6
<i>Panagrellus redivivus</i>	Feeding (killing)	PA01	rhlI/rhlR	PAO1ΔrhlRI	targeted mutation	3	Impaired	6
<i>Panagrellus redivivus</i>	Feeding (killing)	PA01	lasI/rhlR	PAO1ΔlasRI ΔrhlRI	targeted mutation	3	Impaired	6

Table 5.2. [part III] Evidence for the effects of las/rhl quorum sensing disruption on the virulence of experimental infections

Host organism	Infection model	Control strain	Gene(s) disrupted	Strain identity	QS-defective mutant strain	Mutation type	Strain reference	Virulence of QS-defective strain relative to control	Reference
VERTEBRATES									
<i>Danio rerio</i>	Embryonic bloodstream injection	PA14	lasR	?	?	?	?	Impaired	8
<i>Mus musculus</i>	Adult pneumonia (acute)	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	52
<i>Mus musculus</i>	Adult pneumonia (chronic)	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	23
<i>Mus musculus</i>	Burn wound	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	47
<i>Mus musculus</i>	Burn wound	PA14	lasI	PA14Δ <i>lasI</i>	targeted mutation	targeted mutation	45	Impaired	45
<i>Mus musculus</i>	Intraperitoneal sepsis	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	38
<i>Mus musculus</i>	keratitis (scarified)	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	61
<i>Mus musculus</i>	Neonatal pneumonia (acute)	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	41
<i>Mus musculus</i>	Pyelonephritis	PA01	lasI	PAO-JP1	targeted mutation	targeted mutation	40	Impaired	36
<i>Mus musculus</i>	Adult pneumonia (acute)	PA01	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	targeted mutation	32	Enhanced	49
<i>Mus musculus</i>	Burn wound	PA01	lasR	PAO-R1	targeted mutation	targeted mutation	21	Impaired	46
<i>Mus musculus</i>	Burn wound	PA01	lasR	PAO-R1	targeted mutation	targeted mutation	21	Impaired	47
<i>Mus musculus</i>	Burn wound	PA14	lasR	PA14Δ <i>lasR</i>	targeted mutation	targeted mutation	45	Impaired	45
<i>Mus musculus</i>	Burn wound	PA14	lasR	12A1	targeted mutation	targeted mutation	54	Impaired	54
<i>Mus musculus</i>	keratitis (scarified)	PA01	lasR	PAO-R1	targeted mutation	targeted mutation	21	No difference	42
<i>Mus musculus</i>	keratitis (scarified)	PA01	lasR	PAO-R1	targeted mutation	targeted mutation	21	No difference	61
<i>Mus musculus</i>	keratitis (scarified)	ATCC 27853	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	targeted mutation	32	No difference	37
<i>Mus musculus</i>	neonatal pneumonia	PA01	lasR	PAO-R1	targeted mutation	targeted mutation	21	Impaired	55
<i>Mus musculus</i>	Adult pneumonia (chronic)	PA01	rhl	PDO100	targeted mutation	targeted mutation	5	Impaired	23
<i>Mus musculus</i>	Burn wound	PA01	rhl	PDO100	targeted mutation	targeted mutation	5	Impaired	47
<i>Mus musculus</i>	Burn wound	PA14	rhl	PA14Δ <i>rhl</i>	targeted mutation	targeted mutation	45	Impaired	45
<i>Mus musculus</i>	Neonatal pneumonia (acute)	PA01	rhl	PDO100	targeted mutation	targeted mutation	5	Impaired	41
<i>Mus musculus</i>	Burn wound	PA14	rhlR	PA14Δ <i>rhlR</i>	targeted mutation	targeted mutation	45	Impaired	45
<i>Mus musculus</i>	Burn wound	PA14	rhlR	PA14Δ <i>rhlR</i>	targeted mutation	targeted mutation	14	No difference	14
<i>Mus musculus</i>	keratitis (scarified)	PA01	rhlR	PDO111	targeted mutation	targeted mutation	5	No difference	61
<i>Mus musculus</i>	keratitis (scarified)	6294	rhlR	paer2	clinical isolate (corneal infection)	clinical isolate (corneal infection)	60	No difference	61
<i>Mus musculus</i>	keratitis (scarified)	6294	rhlR	paer4	clinical isolate (corneal infection)	clinical isolate (corneal infection)	60	No difference	61

Table 5.2. [part IV] Evidence for the effects of las/rhl quorum sensing disruption on the virulence of experimental infections

Host organism	Infection model	Control strain	Gene(s) disrupted	Strain identity	Mutation type	Strain reference	Virulence of QS-defective strain relative to control	Reference
VERTEBRATES (continued)								
<i>Mus musculus</i>	keratitis (scarified)	6206	las/lasR	paer1	clinical isolate (corneal infection)	60	Impaired	9
<i>Mus musculus</i>	keratitis (scarified)	ATCC 19660	las/lasR	paer1	clinical isolate (corneal infection)	60	Impaired	18
<i>Mus musculus</i>	keratitis (scarified)	6294	las/lasR	paer1	clinical isolate (corneal infection)	60	Impaired	61
<i>Mus musculus</i>	keratitis (scarified)	6294	las/lasR	paer3	clinical isolate (corneal infection)	60	Impaired	61
<i>Mus musculus</i>	keratitis (scarified)	ATCC 19660	las/lasR	paer1	clinical isolate (corneal infection)	60	Impaired	11
<i>Mus musculus</i>	Pyelonephritis	PA01	las/lasR	PA15	clinical isolate (urinary tract infection)	31	Impaired	36
<i>Mus musculus</i>	Adult pneumonia (acute)	PA01	las/rhl	PAO-JP2	targeted mutation	40	Impaired	52
<i>Mus musculus</i>	Adult pneumonia (chronic)	PA01	las/rhl	PAO-JP2	targeted mutation	40	Impaired	23
<i>Mus musculus</i>	Burn wound	PA01	las/rhl	PAO-JP2	targeted mutation	40	Impaired	47
<i>Mus musculus</i>	Neonatal pneumonia (acute)	PA01	las/rhl	PAO-JP2	targeted mutation	40	Impaired	41
<i>Mus musculus</i>	Pyelonephritis	PA01	lasR/rhlR	PAO-JP3	targeted mutation	40	Impaired	36
<i>Mus musculus</i>	Adult pneumonia (acute)	PA01	lasR/rhlR	PA01ΔlasR rhlR	targeted mutation	4	Impaired	4
<i>Mus musculus</i>	Adult pneumonia (chronic)	PA01	lasR/rhlR	PA01ΔlasR rhlR	targeted mutation	4	Impaired	25
<i>Mus musculus</i>	Silicone implant	PA01	lasR/rhlR	PA01ΔlasR rhlR	targeted mutation	4	Impaired	7
<i>Oryctolagus cuniculus</i>	keratitis (intrastromal injection)	ATCC 27853	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	32	No difference	37
<i>Oryctolagus cuniculus</i>	keratitis (scarified)	ATCC 27853	lasR	PA103 (ATCC 29260)	clinical isolate (cystic fibrosis lung)	32	Impaired	37
<i>Rattus norvegicus</i>	Adult pneumonia (acute)	PA01	lasR	PAOR	targeted mutation	27	Impaired	29
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PA01	las/rhl	PAO-JP2	targeted mutation	40	Impaired	59
<i>Rattus norvegicus</i>	Adult pneumonia (chronic)	PA01	las/rhl	PAO-JP2	targeted mutation	40	Impaired	19

References: 1. Alibaud et al. 2008; 2. Attila et al. 2008a; 3. Beatson et al. 2002; 4. Bjarnsholt et al. 2005; 5. Brint and Ohman 1995; 6. G. Campbell, S. P. Diggle et al. (unpublished); 7. Christensen et al. 2007; 8. A. Clatworthy (unpublished, cited in Goldberg et al. 2008); 9. Cole et al. 1998; 10. Cosson et al. 2002; 11. Cowell et al. 1998; 12. Darby et al. 1999; 13. Denervaud et al. 2004; 14. Deziel et al. 2005; 15. S. P. Diggle et al. (unpublished); 16. Erickson et al. 2002; 17. Erickson et al. 2004; 18. Estrellas et al. 2000; 19. Fang et al. 2006; 20. Gallagher and Manoel 2001; 21. Gambello and Iglewski 1991; 22. Hoang et al. 2000; 23. Imamura et al. 2005; 24. Jander et al. 2000; 25. Jensen et al. 2007; 26. Köhler et al. 2000; 27. Latifi et al. 1996; 28. Lau et al. 2003; 29. Lespritt et al. 2003; 30. Lewenza et al. 2005; 31. Liang et al. 2001; 32. Liu 1966; 33. Lutter et al. 2008; 34. Mahajan-Miklos et al. 1999; 35. Matz et al. 2004; 36. Mittal et al. 2006; 37. O'Callaghan et al. 1996; 38. Ozer et al. 2005; 39. Parsons et al. 2002; 40. Pearson et al. 1997; 41. Pearson et al. 2000; 42. Preston et al. 1997; 43. Pukatzki et al. 2002; 44. A. Ross-Gillespie et al. (unpublished); 45. Rumbaugh et al. (submitted); 46. Rumbaugh et al. 1999a; 47. Rumbaugh et al. 1999b; 48. Salunkhe et al. 2005; 49. Sawa et al. 1998; 50. Silo-Suh et al. 2002; 51. Smith et al. 2002a; 52. Smith et al. 2002b; 53. Stoltz et al. 2008; 54. Tan et al. 1999; 55. Tang et al. 1996; 56. This study; 57. Wagner et al. 2004; 58. Walker et al. 2004; 59. Wu et al. 2001; 60. Zhu et al. 2002; 61. Zhu et al. 2004

(Hendrickson et al. 2001; Tan 2002). More generally, it is not uncommon to encounter negative or seemingly contradictory results in the QS literature (Vasil 2003; Kirisits and Parsek 2006; Schuster and Greenberg 2006; Turovskiy et al. 2007).

How can we account for these inconsistent patterns across studies? There are several things to consider. Firstly, and quite obviously, QS is a complex phenomenon. The Las, Rhl and PQS circuits are interconnected in ways that are not yet completely understood, and each circuit is further subject to upstream or downstream modulation by a number of additional regulators that fine tune the QS response (e.g. rpoN, rpoS, qscR, gacA, mvaT and mvfR; see Juhas et al. 2005; Soberon-Chavez et al. 2005; Schuster and Greenberg 2006; Venturi 2006 for further detail). By such secondary regulatory mechanisms, various QS-controlled genes, including the rhl circuit, may still be active in las mutants (Venturi 2006). Alternatively, las mutants can sometimes harbour compensatory mutations (e.g. affecting pilus formation) that could contribute to virulence (Beatson et al. 2002). Also, it is important to remember that QS regulation can repress, as well as promote, target genes (Schuster et al. 2003; Wagner et al. 2003). Thus, to think of las mutants as being devoid of QS-activity and universally repressed for QS-associated traits would be a naïve oversimplification of a complex reality.

Secondly, both the timing and intensity of QS expression are highly sensitive to environmental variation, such as medium composition, alkalinity or oxygen, so even small changes in environmental conditions could potentially have a profound effect on metabolism and pathogenesis (Bazire et al. 2005; Shrout et al. 2006; D'Argenio et al. 2007; Diggle et al. 2007b; Duan and Surette 2007). For example, *P. aeruginosa* can kill nematodes by several completely distinct processes, which may or may not involve QS, depending on the growth medium (Tan 2002). Similarly, within the host, different types of tissue present dramatically different challenges to bacteria and, indeed, different infection routes into the same host organism also vary in their dependence on QS (Rumbaugh et al. 2000; Lutter et al. 2008; Willcox et al. 2008).

Thirdly, different studies have employed different strains of *P. aeruginosa*. Substantial genotypic and phenotypic differences exist between the two most widely used strains, PA01 and PA14 (Choi et al. 2002; Lee et al. 2006; D'Argenio et al. 2007; Carilla-Latorre et al. 2008; Kukavica-Ibrulj et al. 2008) and even within the PA01 type strain, adaptation to local laboratory conditions could rapidly generate differences between isolates under study at different laboratories (Fux et al. 2005). It should not be surprising then, to find differences in pathogenesis among different strains.

Fourthly, infection models for bacterial pathogenesis necessarily focus on the variation due to the pathogen but it may be misleading to assume that host-responses are invariant across replicates and across experiments. QS-controlled virulence factors, or even QS-signals themselves, can have strong immunomodulatory effects, which can profoundly influence the resultant virulence of an infection (Hooi et al. 2004; Pritchard 2006). Host immune responses, and thus infection outcomes, are also known to vary temporally (e.g. Lee and Edery 2008), and spatially (e.g. Corby-Harris and Promislow 2008), and can depend on prior immune challenges (Apidianakis et al. 2005), yet such variables are not always adequately controlled for, or even reported, in laboratory studies.

Finally, for a more holistic picture of the role of QS in pathogenesis, we may need to consider both acute and chronic settings. In this study, using acute infection models optimized for rapid bacterial growth, we found limited evidence of a role for QS in pathogenesis. Perhaps, however, QS is more important in a chronic infection context (Nguyen and Singh 2006), for example, because of its role in biofilms (Kirisits and Parsek 2006). Alternatively, QS-mediated motility could allow migration from the site of infection, and so facilitate the establishment of a systemic infection (Glessner et al. 1999; Köhler et al. 2000; Lesic et al. 2007). On the other hand, there are key QS-controlled processes, such as type III secretion or immunomodulation, which may be most important early in the infection process (Bjarnsholt et al. 2005; Lee et al. 2005a; Willcox et al. 2008). Early transcriptional studies reported that QS mutants do not grow faster under standard laboratory conditions (Heurlier et al.

2005; An et al. 2006), and that QS-regulated genes were expressed only once a population has reached stationary phase (Whiteley et al. 1999; Schuster et al. 2003; Heurlier et al. 2005). More recently, however, it has been shown that QS can function during the growth phase too (i.e. in response to a lower quorum), depending on the selective conditions (Alvarez-Ortega and Harwood 2007; Duan and Surette 2007; Sandoz et al. 2007), so QS may be involved in both acute and chronic infections, depending on the model.

The discovery that QS affects bacterial virulence *in vivo* in multiple host models prompted much excitement: *P. aeruginosa* is notoriously difficult to control using conventional antibiotics, and here, finally, was a promising target for novel therapies (Donabedian 2003; Cegelski et al. 2008). Much attention was (and is) focused on developing new ways to disrupt QS (“quorum quenching”; Hentzer et al. 2003; Smith et al. 2003; Xu et al. 2003; Roche et al. 2004; Zhang and Dong 2004; Rice et al. 2005; Gonzalez and Keshavan 2006; Rasmussen and Givskov 2006; Lesic et al. 2007). An alternative approach could be to harness the social side of QS (Foster 2005). In light of our demonstration that QS functions as a cooperative trait in a mouse infection model (Rumbaugh et al., submitted), we recently raised the possibility that QS cheats could be introduced into hosts to outcompete wild type cooperators. The present study, however, highlights important limitations in either approach. QS is not universally required for growth *in vivo*, nor is growth *in vivo* necessarily correlated with virulence, nor are all virulence factors under QS-regulation, so disrupting QS may not always attenuate virulence (Table 5.2). Increasing the proportion of QS-deficient mutants, therefore, may not always act to weaken the bacterial population: QS-defective mutants can, under certain conditions, have an absolute (as opposed to relative) fitness advantage over QS-proficient cells (Heurlier et al. 2005; D'Argenio et al. 2007; Diggle et al. 2007b). Indeed, there is much we still need to learn about both the proximate (molecular) mechanisms by which QS operates and the ultimate (evolutionary) forces by which it is maintained.

6. General discussion and concluding remarks

Each of the four preceding chapters of this thesis has included its own extensive discussion. In this final chapter I summarise the findings from each of these chapters and make some general observations on the progress that has been made and the implications arising from this work.

In **chapter 2**, I explored the question of when and why cooperation should be frequency dependent – essentially the idea that cheats prosper most when rare. Despite its intuitive appeal, Hamilton (1964) demonstrated that frequency dependence is actually not predicted to occur under most circumstances because, even though cheats do benefit from a relative profusion of cooperators, cooperators also benefit from this situation since their cooperation becomes more efficient. In contrast with this theoretical expectation that frequency dependent cooperation should be rare, several recent studies have demonstrated that the phenomenon does evidently occur in microbes (Velicer et al. 2000; Vulic and Kolter 2001; Fiegna and Velicer 2003; Rainey and Rainey 2003; Turner and Chao 2003; Dugatkin et al. 2005; Brockhurst et al. 2006; Harrison et al. 2006; MacLean and Gudelj 2006; Diggle et al. 2007; Ellis et al. 2007; Gilbert et al. 2007).

To clarify this situation and account for the apparent mismatch between existing models and data, we used individual based kin selection methodology to model public goods cooperation in a microbial context. It has been argued before that frequency dependent cooperation can arise in situations with strong selection (Charlesworth 1978; Michod 1982; Toro et al. 1982; Queller 1984; Nowak and May 1992; Day and Taylor 1998; Rousset 2004; Jansen and Van Baalen 2006). Our models reveal the additional requirements that either (a) cooperators and cheats do not share the same social environment (structured population), or (b) a population's growth is a function of its composition of cooperators and cheats (frequency dependent population growth). I tested the last of these predictions in laboratory experiments with *Pseudomonas aeruginosa*. As predicted, I found that cheats had greater relative fitness when rare, and that this effect arose because populations with mostly cheats were less productive than populations in which cheats were rare. These

theoretical results, backed up with empirical support, advance our understanding of when and why we observe frequency dependent cooperation in microbes.

In a similar fashion to the previous chapter, the work in **chapter 3** employed a combination of theory and empirical experiments to investigate the conditions under which selection for cooperation is expected to vary with population density. Density-dependence has not been as well explored as frequency dependence by empiricists, and two recent studies, both of yeast, have reported opposing results. Greig and Travisano (2004) reported that cheats exploited cooperators best when population density was high; while (MacLean and Gudelj 2006; MacLean 2008) found the opposite pattern, with cheats performing poorly relative to cooperators at higher densities.

By highlighting the distinction between two different forms of cooperative dilemma (public goods vs. tragedy of the commons), and then explicitly modeling either scenario, we were able to resolve this apparent conflict. Our models predict that under public goods type cooperation, cheats will have a higher relative fitness at higher population densities because they have a greater access to the public goods produced by cooperative neighbours, and also because whole-group relatedness is decreased under these conditions. In contrast, under tragedy of the commons (restraint) type cooperation, cheats can have either a higher or a lower relative fitness at higher population densities depending on the particular parameter values of the model (i.e. system-specific biological details). I used siderophore production by *P. aeruginosa* to empirically evaluate our theoretical predictions for a public goods type cooperative scenario. My results confirmed that cheat relative fitness was greater at higher population densities, as predicted, and that this effect arose, at least in part, because the closer proximity between cooperators and cheats (as would occur at high densities) allowed cheats better access to the public good. This study resolves an apparent conflict in the literature and brings greater clarity to this area of social evolution theory.

In chapters 4 and 5, I tested the idea that social conflict between cooperators and cheats, in the context of an infecting population of pathogens, could attenuate population productivity and hence decrease the virulence of the infection (Brown et al 2002; West and Buckling 2003). This hypothesis is important because the effect it predicts (lower virulence at higher multiplicity of infection) contrasts directly with that of classic virulence theory (Frank 1996; Frank 1998; Read and Taylor 2001). This theory therefore has potential implications for the medical management of virulence but, to date, it has received scant empirical attention.

In **chapter 4**, as in the preceding chapters, I used siderophore production by *P. aeruginosa* as my model trait, in experimental infections of plant and insect hosts. In contrast to previous work by Harrison et al. (2006), where they did find, as expected, attenuation of bacterial growth and virulence in co-infections with siderophore producers and non-producers, here I found no fitness benefit for siderophore production *in vivo*. Consequently, I also found no evidence that non-producers gained relative benefits through exploiting producers in co-infection. In summary, I was unable to find support for the hypothesis of West and Buckling (2003).

For social conflict to play any significant role in the demography of a population, it is necessary first that the social costs and benefits are associated with the expression or non-expression of the putatively social trait. Despite the supposed importance of siderophore production to *P. aeruginosa* iron metabolism (Visca et al. 2007), surprisingly few studies have rigorously tested for the *in vivo* consequences of knocking out siderophore production in this pathogen - particularly in non-mammalian hosts. From the evidence that is available (Table 4.1), it seems clear that siderophores are crucial to infections in mammalian tissues; however, there is little evidence of an important role in virulence in plant hosts. In such contexts, bacteria defective for siderophore production may well be able to acquire adequate iron via alternative pathways, without recourse to siderophores. Thus, while siderophore production can function as an important social trait *in vitro* (Griffin et al. 2004; Buckling et al. 2007; Ross-Gillespie et al. 2007), the importance of such dynamics in real infections may be highly dependent on the particular host and infection context.

Another trait thought to be highly important in bacterial virulence is quorum sensing (Donabedian 2003) and recent work has demonstrated that this can be a social trait, subject to exploitation by cheats (Diggle et al. 2007; Sandoz et al. 2007). In **chapter 5**, I adopted the same experimental protocols developed in the previous chapter to investigate the potential for social conflict in this trait *in vivo*, and its potential effects on virulence. I constructed knockout strains defective for either the production or reception of quorum sensing signals, and tested them in single and mixed infections of insects and plants. My data indicate that quorum sensing did not provide a bacterial growth advantage in these infection models and accordingly, there was no consistent evidence of social exploitation among strains. Thus, I found no support for the idea that QS functions as a cooperative trait *in vivo* in these hosts.

In my experiments, infections with QS-defective strains did not consistently show attenuated virulence, despite the supposed importance of this trait to pathogenesis (Donabedian 2003). In contrast to the situation with siderophores, a considerable number of studies have now tested for the virulence consequences of disrupted QS in *P. aeruginosa* and I conducted a comprehensive survey of the literature on this subject (Table 5.2). Though the majority of studies support the idea that disruption of QS leads to attenuated virulence, exceptions (including my own experiments presented in chapter 5) have been reported, and I conclude that it is misleading to ascribe universal control of virulence to this highly complex trait. Instead, work in this area must take into consideration the finer biological details that determine when, how and to what extent QS influences virulence.

In chapters 2, 3 and 4 of the thesis, I employed the same three strain pairs to investigate cooperator/cheat dynamics in the context of siderophore production under various conditions. My experiments revealed significant qualitative differences across these strain pairs, in terms of the strength and direction of frequency- and/or density dependence, and in the relative virulence and *in vivo* growth of cooperators vs. cheats. Let us now briefly consider whether these differences were consistent across experiments and, if so, what possible features of these particular strain pairs could have given rise to these differences. Strain pair B – in which the difference

between B+ and B- is a single gene mutation – conformed well to our theoretical predictions, except that we saw little difference between B+ and B- in terms of *in vivo* growth and virulence. The performance of strain pair C, meanwhile, matched our expectations fairly well under *in vitro* conditions (which were broadly similar to the conditions under which C+ and C- coevolved for the past 100+ generations) but, *in vivo*, we found that C- produced a more virulent infection. This suggests that C-, despite its defective siderophore production, has acquired additional mutations that make it more harmful to hosts. Strain pair A has been used previously in social evolution studies of siderophore production (Griffin et al. 2004; Harrison et al. 2006), and for the most part it performed as predicted by theory. Unexpectedly, however, our data show that A- (PA09) does not show positive density dependent fitness. I suspect this may be due to additional defects this strain carries in its QS signalling pathways that, under the conditions of our experiment, act to reduce its fitness relative to the wildtype at densities where QS would normally be activated (for instance, quinolone signalling appears to be defective in this strain; Steve Diggle, unpublished data). The fact that these striking differences still arose in strain pairs selected on the basis of their supposed similarities (each comprised a siderophore proficient and a non-proficient strain), underscores the value of studying functional traits in multiple independent strains. In the remainder of this discussion, I consider some general themes that warrant consideration by workers in the field of sociomicrobiology.

Adaptationist vs. mechanistic approaches

Social evolution, and behavioural ecology more generally, employs an adaptationist approach that assumes that the evolution of behaviours can be understood from a purely phenotypic perspective. This approach, termed ‘the phenotypic gambit’ (Grafen 1984), although it mostly ignores underlying genetic architecture, has served us well and through it we have come to learn much about the evolutionary function of behavioural traits. Indeed, sometimes the details don’t matter. In chapter 2, for instance, our prediction that cheat fitness should be negatively frequency dependent was upheld for three different strain pairs, derived independently and featuring different mutations (Figure 2.1).

In other situations, however, the details do matter. Host-pathogen interactions are highly complex and involve numerous molecular pathways. The phenotypic gambit should be relatively safe to apply to behaviours at long-term evolutionary equilibria, because, given enough time, traits should tend towards being optimally adapted, irrespective of the underlying genetic architecture. In the context of virulence, meanwhile – especially experimental infections involving hosts and parasites that have not shared a long history of co-evolution – we should proceed with caution and pay more attention to detail (Buckling and Brockhurst 2008).

General theories describing the evolution of virulence have not been very successful in accounting for observed patterns (Herre 1993; Frank 1996; Gandon et al. 2001; Read and Taylor 2001; Griffin and West 2002), and even when some of the details are known (for example, that the *lasR* gene is required for 3-oxo-C12-HSL-mediated quorum sensing), other system-specific details (interactions between gene regulatory networks, host immune responses, etc.) can still obscure patterns. If evolutionary biology is to make a meaningful contribution to our understanding of when and why some infections are virulent while others are benign, we will need to complement our (laudable) quest for minimalist models that capture general patterns with a willingness to engage more with the detail (Frank and Schmid-Hempel 2007; Schmid-Hempel and Frank 2008).

More generally, to reveal whether or not phenomena of interest will vary from one situation to another, it is important that we replicate experiments, with different pathogen strains, in different hosts, and in different laboratories, in order to test the generality of our theories. Indeed, I have already alluded to this issue above in my discussion of the inter-strain variation seen in my experiments. There is an alarming tendency among cell biologists to under-replicate, or pseudoreplicate, experiments and draw inferences from dubious statistics. Evolutionary biologists meanwhile, although they come from a stronger tradition of implementing appropriate statistics and experimental design methodologies *within* studies, seldom deem it worthwhile to replicate the same experiments across different laboratories, and so might more often fail to notice important but unforeseen “minor” details.

What is virulence?

There is a certain amount of confusion, generally, about what the term ‘virulence’ actually means (Read 1994; Casadevall and Pirofski 1999; Poulin and Combes 1999; Casadevall and Pirofski 2001). In medical fields, and in microbiology, virulence is regarded as the ability of a pathogen to multiply and cause disease in a host (i.e. growth, persistence, invasion, production of virulence factors). This broad definition either ignores or conflates adaptations of the host that resist damage by the pathogen. Evolutionary biologists, meanwhile, tend to define virulence strictly in terms of the pathogen-induced fitness effects on the host (i.e. mortality, reduced reproductive output) when in reality they are trying to indirectly capture some underlying attribute of the pathogen (I have adopted this convention in this thesis too). Either approach is problematic since it measures one side of the interaction only. What we are actually referring to when we speak of virulence is an emergent property of the interaction between a particular host and a particular pathogen at a particular time.

Though this may seem an unimportant semantic issue, it highlights an important distinction (Read 1994). Consider for example, what exactly we mean by “virulence genes”? Are these genes that act to enhance pathogen productivity, or host damage? One pattern to emerge from my experiments in chapters 4 and 5, and from my surveys of the literature (Tables 4.1 and 5.2), is that damage to a host (i.e. host fitness) is not necessarily correlated with bacterial growth (pathogen fitness), as is sometimes asserted in the microbiological literature, and as would be expected if these traits were perfectly co-adapted over long periods of time.

This decoupling of host fitness and bacterial fitness is significant: therapies designed to attenuate virulence (i.e. minimize damage to a host) without affecting pathogen fitness may be more effective than strategies which seek to attenuate virulence by thwarting pathogen growth, because in the latter scenario, pathogens would be under strong selection to evolve resistance to the therapy (Cegelski et al. 2008). To better understand the evolution of virulence, future studies should, wherever possible, collect data on the fitness consequences of an infection to both pathogen and host.

Social traits and pathogen control

The two traits I have studied in this thesis (siderophore production and QS) have great significance for the biology of *Pseudomonas aeruginosa*, and indeed microbes in general, and accordingly have attracted much attention in the field of applied microbiology. Since iron is crucial for pathogens, iron uptake pathways are obvious targets for antibacterial drugs. Against a backdrop of increasingly widespread resistance to conventional antibiotics – among microbes in general (Neu 1992; Alanis 2005; Livermore 2005; Thomson and Bonomo 2005; Payne 2008) and in the case of *P. aeruginosa* in particular (Lambert 2002; Livermore 2002) – there is much current interest in finding novel ways of blocking iron metabolism (Miethke and Marahiel 2007), or potentially hijacking siderophore uptake mechanisms to introduce antibiotics directly into the bacterial cytoplasm (Budzikiewicz 2001). Similarly, there has been, and is, much interest in pharmacologically disrupting QS (Hentzer et al. 2003; Smith et al. 2003; Xu et al. 2003; Zhang and Dong 2004; Rice et al. 2005; Gonzalez and Keshavan 2006; Rasmussen and Givskov 2006; Lesic et al. 2007).

At the same time, considering the ubiquity of social behaviour in microbial life (Crespi 2001; West et al. 2006; Frank 2007; West et al. 2007), it may be useful to look to social evolution for inspiration for alternative, complementary therapies. Although my experiments (chapters 4 and 5) failed to find support for a significant role of social conflict *in vivo*, such a role has been found in other studies (Harrison et al 2006; Rumbaugh et al, submitted) and substantial circumstantial evidence from *in vitro* studies (Buckling et al. 2007; Diggle et al. 2007) suggests that we should anticipate that, in some circumstances at least, social conflict among pathogens could be very important in determining the course of an infection. Manipulations that change the costs and benefits of cooperation and cheating could potentially have a profound influence on the productivity and virulence of a pathogen population (“Hamiltonian medicine”; Foster 2005; André and Godelle 2005). It is an exciting prospect: the impressive sociality of pathogens may indeed hold the key to their downfall.

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Appendices

- A1: Photographs illustrating strain-specific colony morphotypes for the three *Pseudomonas aeruginosa* strain pairs featured in chapters 2, 3, and 4 of this thesis.
- A2: Ross-Gillespie, A., Gardner, A., West, S.A. and A. S. Griffin. 2007. Frequency dependence and cooperation: theory and a test with bacteria. *American Naturalist* 170:331-342.
- A3: Ross-Gillespie, A., O'Riain, M. J. and L. F. Keller. 2007. Viral epizootic reveals inbreeding depression in a habitually inbreeding mammal. *Evolution* 61:2268-2273.
- A4: Ross-Gillespie, A. and A. S. Griffin. 2007. Quick Guide: Meerkats. *Current Biology* 17:442-443.

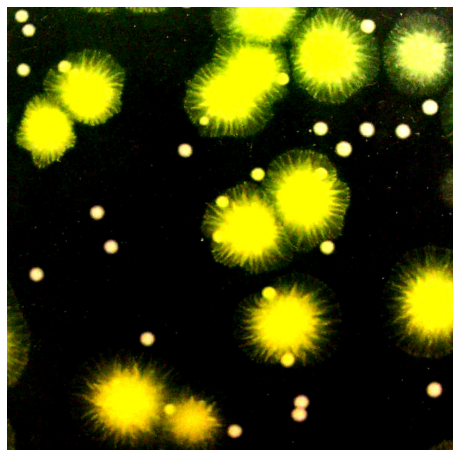
Appendix A1

This plate illustrates the contrasting colony morphologies of the wild-type cooperator strain (green; siderophore producing) and mutant cheat strain (white; siderophore-defective) from each of three strain pairs featured in chapters 2, 3 and 4 of this thesis. For these photographs, mixed cultures of wild-type and mutant cultures were plated to Kings B agar and incubated overnight at 37°C. Further details on strain sources and mutagenesis procedures are provided in Chapter 2.

Strain pair A

Wild type:
PA01 ATCC15692

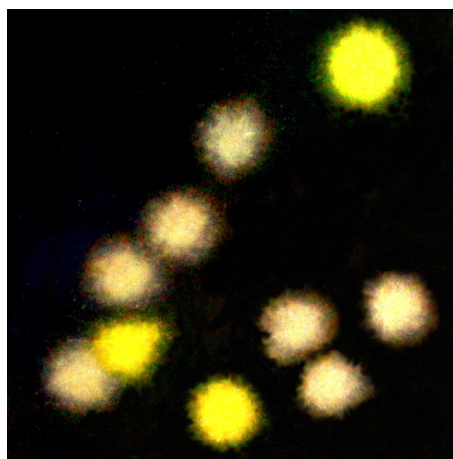
Mutant:
PAO6609, derived by UV-mutagenesis from
methionine auxotroph PAO6409



Strain pair B

Wild type:
PA01 ATCC15692

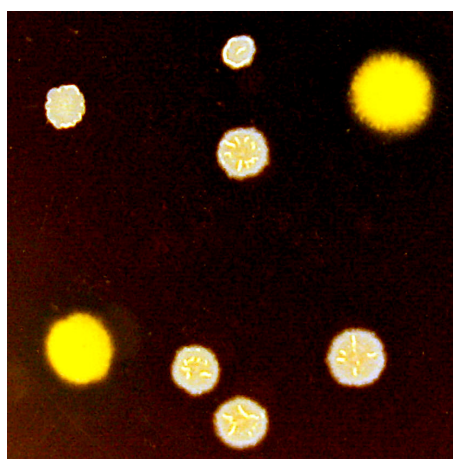
Mutant:
PA01 $\Delta pvdD$



Strain pair C

Wild type:
“7F+”, laboratory-selected derivative of
UCBPP-PA14

Mutant:
“7K-”, spontaneous mutant isolated from the same
experimentally-evolved laboratory culture as 7F+



Frequency Dependence and Cooperation: Theory and a Test with Bacteria

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Submitted December 4, 2006; Accepted April 23, 2007;
Electronically published July 19, 2007

ABSTRACT: Hamilton's inclusive fitness theory provides a leading explanation for the problem of cooperation. A general result from inclusive fitness theory is that, except under restrictive conditions, cooperation should not be subject to frequency-dependent selection. However, several recent studies in microbial systems have demonstrated that the relative fitness of cheaters, which do not cooperate, is greater when cheaters are rarer. Here we demonstrate theoretically that such frequency-dependent selection can occur in microbes when there is (1) sufficient population structuring or (2) an association between the level of cooperation and total population growth. We test prediction (2) and its underlying assumption, using the pathogenic bacterium *Pseudomonas aeruginosa*, by competing strains that produce iron-scavenging siderophore molecules (cooperators) with nonproducers (cheaters) at various ratios, under conditions that minimize population structuring. We found that both the relative fitness of cheaters and the productivity of the mixed culture were significantly negatively related to initial cheater frequency. Furthermore, when the period of population growth was experimentally shortened, the strength of frequency dependence was reduced. More generally, we argue that frequency-dependent selection on cooperative traits may be more common in microbes than in metazoans because strong selection, structuring, and cooperation-dependent growth will be more common in microbial populations.

Keywords: *Pseudomonas aeruginosa*, public goods, siderophores, strong selection, kin selection, population structure.

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Explaining cooperation is one of the greatest challenges for evolutionary biology (Maynard Smith and Szathmàry 1995; Hamilton 1996). The problem is this: why should an individual perform a cooperative behavior that appears costly to perform but benefits other individuals (Hamilton 1964)? Following a wealth of theoretical work in this area in recent decades, we now have an excellent general understanding of the different ways in which cooperation can be favored (reviewed by Frank [2003]; Sachs et al. [2004]; Lehmann and Keller [2006]; West et al. [2006], [2007]). However, much of this vast body of theory has been developed to elucidate general principles and is less useful for stimulating empirical tests of theory (Leimar and Hammerstein 2006). Consequently, a major task for social evolution research is to take this general theory and develop it so it can be tested empirically, with specific groups of organisms.

Microbes offer a number of advantages for testing social evolution theory (reviewed by Crespi [2001]; West et al. [2006]). In particular, they perform a number of cooperative behaviors, where it is possible to alter the costs and benefits of cooperation experimentally and then follow the fitness consequences (West et al. 2006). Recently, there has been interest in how the relative fitness of cooperators, and cheaters who do not cooperate (or cooperate less), depends on the relative proportion of cooperators in the population (Levin 1988; Velicer et al. 2000; Dugatkin et al. 2003, 2005; Harrison et al. 2006; MacLean and Gudelj 2006). However, these experimental examinations of frequency dependence contrast with most theory in this area, where frequency dependence is not predicted. Indeed, a major result from Hamilton's (1964) original inclusive fitness formulation, showing how cooperation can be favored between relatives, was that the inclusive fitness of cooperators did not vary with gene frequency at all. Instead, because increased cooperation provides benefits to both cooperators and cheaters, the inclusive fitness advantage of cooperation remains constant despite changes in the frequency of cooperators. Hamilton was so pleased by this

result that he later described it as a “gift from God” (Hamilton 1988, p. 16). Subsequent work has shown that frequency dependence can enter into kin selection theory in cases featuring strong selection (genes with large fitness effects) or nonadditivity (e.g., Charlesworth 1978; Michod 1982; Toro et al. 1982; Queller 1984; Day and Taylor 1998; Frank 1998; Rousset 2004; Wild and Traulsen 2007).

Our first aim in this article is to develop theory that predicts specifically when and why frequency-dependent selection on cooperation should be observed in microbes. The phenomenon is illustrated by considering the extracellular secretion of substances that can be utilized by neighboring cells. The production of such “public goods” requires explanation because they are costly to the individual to produce but provide a benefit to the local group (West et al. 2006). In this situation, we might, at first glance, expect that selection on cells that contribute less than their fair share to the public good resource pool (cheaters) should be frequency dependent. That is, cheaters should do better when they are rare, because there will be more public goods/cooperators for them to exploit. However, cooperators also gain a benefit from being in a more cooperative population. In the simplest scenario, these two effects exactly cancel, and so selection on cheaters and cooperators does not depend on their frequency (see next section). We examine the extent to which frequency dependence does arise when the following biological complexities are taken into consideration: population structure, effects of cooperation on population growth, and large (as opposed to small) fitness consequences associated with variation in the level of cooperation (strong selection).

Our models predict that, given strong selection, cheaters will be subject to negative frequency-dependent selection (i.e., their relative fitness will be lower when common) when (1) the population is structured and/or (2) a higher frequency of cooperators leads to greater population growth. We tested prediction (2) and the underlying assumption on the relationship between growth and cooperator frequency by investigating the production of a public good, iron-scavenging siderophore molecules, in the bacterial pathogen *Pseudomonas aeruginosa* (West and Buckling 2003; Griffin et al. 2004). Iron is a major limiting factor for bacterial growth, because most iron in the environment is in the insoluble Fe(III) form and is actively withheld by hosts (Guerinot 1994; Ratledge and Dover 2000). Siderophores scavenge insoluble and host-bound iron, making it available for bacterial metabolism. To determine whether frequency dependence is a general characteristic of *P. aeruginosa* siderophore production and not just a pleiotropic effect associated with a specific cooperator-cheater pair, we examined three independently derived cooperator-cheater strain pairs. These involved mu-

tants produced by ultraviolet mutagenesis, single-gene deletion, and spontaneous natural mutation, respectively.

Models and Analyses

We explore some ways in which frequency-dependent selection can arise in kin selection models for the production of public goods dilemmas in microbes. In particular, we examine the role of (1) population structure (including mixing rates and the scale over which social interaction occurs) and (2) the dependence of population carrying capacity and growth on the level of public goods contributions. In both cases, we allow for strong selection, which is crucial for frequency dependence in the context of social behaviors and is an important characteristic of many microbial cooperative traits (see “Discussion”). To begin, we demonstrate that frequency dependence does not emerge under the simplest possible scenario with weak selection.

Weak Selection and Frequency Independence

In a broad class of models, weak selection leads to frequency independence (Rousset 2004, p. 80; 2006). To see why, consider a large population facing a public goods dilemma, such as, for example, siderophore production. An individual's fitness is a function of (1) its level of public goods contribution, s , and (2) the average public goods production across the whole population, \bar{s} . Without making further assumptions, we can express fitness as $w(s, \bar{s})$; this can be done for structured and unstructured populations. Further, we assume that cooperative individuals contribute an amount s_C and that a proportion p of the individuals in the population are defectors (cheaters) who contribute a smaller amount, $s_D = s_C - \delta$; hence, $\bar{s} = s_C - p\delta$. Thus, the fitness of a cooperator is

$$w_C = w(s_C, s_C - p\delta), \quad (1)$$

and the fitness of a cheater is

$$w_D = w(s_C - \delta, s_C - p\delta). \quad (2)$$

The relative fitness of cheaters is given by the ratio of cheater and cooperator fitness and, using a Taylor expansion, can be expressed as

$$v_D = \frac{w_D}{w_C} = 1 - \left. \frac{\partial w}{\partial s} \right|_{\delta=0} \delta / \hat{w} + O(\delta^2), \quad (3)$$

where $\hat{w} = w(s_C, s_C)$ is the fitness of cooperators in the absence of cheaters. This reveals that, to leading order in δ , the relative fitness of cheaters is not a function of their

frequency p in the population (the partial derivative appearing in eq. [3] is not a function of p , because it is evaluated in the neutral population where $\delta = 0$, so that cooperators and defectors are indistinguishable and the frequency of the latter is irrelevant). Hence, if selection is weak (δ sufficiently small for higher-order terms in δ to be negligible; the δ -weak selection of Wild and Traulsen [2007]), it is also frequency independent. For stronger selection, the trailing term of order δ^2 becomes more important, and if this is a function of the frequency of cheaters, then there is the possibility of frequency-dependent selection. Note that although our analyses focus on whether the fitness of cheaters is frequency dependent, the fact that we are examining relative fitness means that they also demonstrate the nature of selection on cooperators—if the relative fitness of cheaters decreases as they become more common, then the relative fitness of cooperators must increase as they become rarer.

Structured Populations

One way in which strong selection can give rise to frequency-dependent selection is if populations are structured so that cooperators and cheaters do not share the same social environment. This could occur in populations with localized social interaction due to limited mixing of public goods or cells. A consequence of this is that more cooperative individuals would have greater access to public goods, either because they enjoy preferential access to the public goods that they have produced or because they use the public goods produced by their clonal relatives (who are also cooperators). We examine this in general terms, describing fitness as a product of two components that capture (1) the cost of producing public goods and (2) the benefit of receiving public goods. In particular, we write

$$w(s, \hat{s}) = g(s)h(\hat{s}), \quad (4)$$

where w is fitness, g is a growth component that decreases with one's individual allocation to public goods production s , and h is a growth component that increases with one's access to public goods \hat{s} (for related microbial models, see Brown 1999; Smith 2001; West et al. 2002; Dugatkin et al. 2003; West and Buckling 2003). In mathematical terms, we have $g > 0$, $\partial g / \partial s = g' < 0$, $h > 0$, and $\partial h / \partial \hat{s} = h' > 0$. We make the further assumption of linear or diminishing returns to fecundity as access to public goods in the social environment is increased, giving $\partial^2 h / \partial \hat{s}^2 \leq 0$. Due to population structure, the level of public goods available in the social environment is correlated with the individual's own allocation to public goods, according to the expression $\hat{s} = rs + (1 - r)\bar{s}$; that is, the

level of public goods available lies between one's own investment (s) and the population average investment (\bar{s}), according to the relatedness between social partners (r). The variable r encapsulates the effects of both bacterial dispersal and the diffusion of public goods through the population. It therefore allows us to move between the extremes of a completely mixed population or global diffusion of public goods ($r = 0$) and a completely structured population, where all neighbors are of the same genotype or public goods disperse over such small distances that they are utilized only by the individual that produced them ($r = 1$; cheaters only interact with cheaters, and cooperators only interact with cooperators).

We compare the fecundity of a cheater strain that allocates nothing to public goods ($s = 0$, defection) to that of a strain that allocates a standard unit ($s = 1$, cooperation). Respectively, these are

$$w_D = g(0)h[(1 - r)(1 - p)] = g_D h_D \quad (5)$$

and

$$w_C = g(1)h[r + (1 - r)(1 - p)] = g_C h_C, \quad (6)$$

where p is the population frequency of cheaters, and hence $\bar{s} = 1 - p$ is the population average allocation to public goods, being the proportion of cooperators. The relative fitness of cheaters can be written as

$$v = \frac{w_D}{w_C} = \frac{g(0)h[(1 - r)(1 - p)]}{g(1)h[r + (1 - r)(1 - p)]} = \frac{g_D h_D}{g_C h_C}. \quad (7)$$

We now determine whether and how the relative fitness of cheaters (v) varies with their frequency in the global population (p):

$$\frac{dv}{dp} = \frac{g_D}{g_C}(1 - r) \frac{h_D h'_C - h_C h'_D}{h_C^2}. \quad (8)$$

This reveals the following: (1) in the absence of population structure ($r = 0$), there is no frequency dependence ($dv/dp = 0$), because cooperators and cheaters share the same social environment; hence, $h_C = h_D$ and $h'_C = h'_D$; (2) in fully structured populations ($r = 1$), there is no frequency dependence ($dv/dp = 0$), because an individual's social environment is dependent only on its own cooperation strategy and not on the global frequency of cooperators; (3) in a population with intermediate structuring ($0 < r < 1$), there is negative frequency dependence ($dv/dp < 0$), because cooperators enjoy more public goods in their social environment than do cheaters and hence $h_C > h_D$ and $h'_C \leq h'_D$.

The impact of population structure (r) on cheater rel-

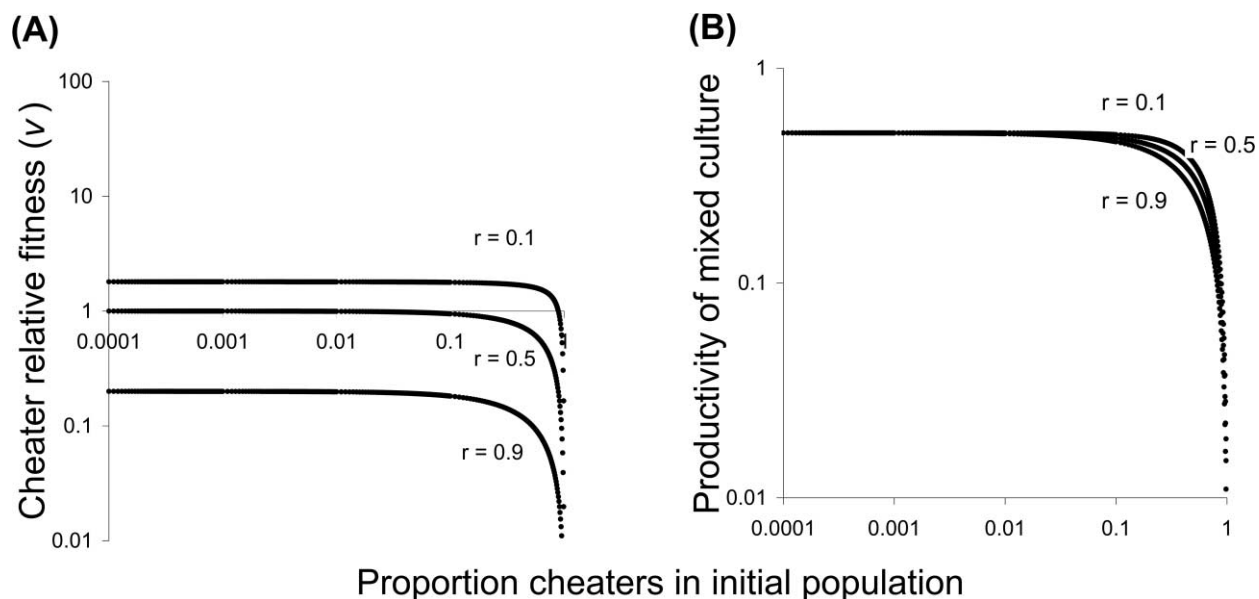


Figure 1: Structured population model, with fitness given by the product of $g(s) = 1 - s/2$ and $h(\hat{s}) = \hat{s}$, where s is an individual's own contribution to public goods and \hat{s} is the amount of public goods in the individual's social environment. *A*, Relative fitness of cheaters (v) decreases with their frequency (p) and with the degree of population structure (r). *B*, Total productivity of the population decreases with the frequency of cheaters (p) and with degree of population structure (r).

ative fitness is examined using the same approach. The derivative

$$\frac{dv}{dr} = -\frac{g_D p h_D h'_C + (1-p) h_C h'_D}{h_C^2} \quad (9)$$

is a negative quantity, and hence increasing population structure (higher r) reduces the relative fitness of cheaters. This is because cheaters will have a higher fitness in more mixed populations, where they are better able to exploit the cooperators. This could be tested empirically by examining the strength of frequency dependence across populations that are structured to different degrees, for example, using shaken versus unshaken liquid culture media or semisolid agar culture media of varying viscosities. Numerical illustrations of the model are given in figure 1.

Population Growth as a Function of Cooperation Frequency

Another way in which strong selection can give rise to frequency dependence is when the growth of a population depends on its genetic composition. If the growth of a bacterial population is negligible when cheating is prevalent, we expect little change in population size and genetic composition, due to cheaters having a limited opportunity to exploit cooperators. Conversely, if more cooperative

bacterial colonies achieve a higher carrying capacity and hence more growth, differences in growth rates of cooperators and cheaters could lead to more pronounced changes in gene frequencies. Describing fitness in terms of absolute increase over the growth period, the relative fitness of faster growers increases with the number of rounds of division. Put simply, more growth means a greater chance for cheaters to exploit cooperators.

For simplicity, consider a well-mixed population of bacteria growing exponentially over a time period that depends on the initial proportion of cooperators. Defining a basic time unit such that the instantaneous rate of growth is 1 for cooperators, then the growth rate of cheaters can be represented as $1 + b$. After t time units, the numbers of cooperators and cheaters are given by

$$n_{C,t} = n_{C,0} e^t, \quad (10)$$

$$n_{D,t} = n_{D,0} e^{(1+b)t}, \quad (11)$$

respectively, where $n_{C,0}$ and $n_{D,0}$ are, respectively, the numbers of cooperators and cheaters at time $t = 0$. If growth ceases at time $t = T(p_0)$, which decreases with the initial proportion of cheaters (p_0 ; i.e., $dT/dp_0 < 0$), then the frequency of cheaters following growth is given by

$$p_T = \frac{n_{D,T}}{n_{D,T} + n_{C,T}} = \frac{p_0 e^{bT}}{p_0 e^{bT} + 1 - p_0}. \quad (12)$$

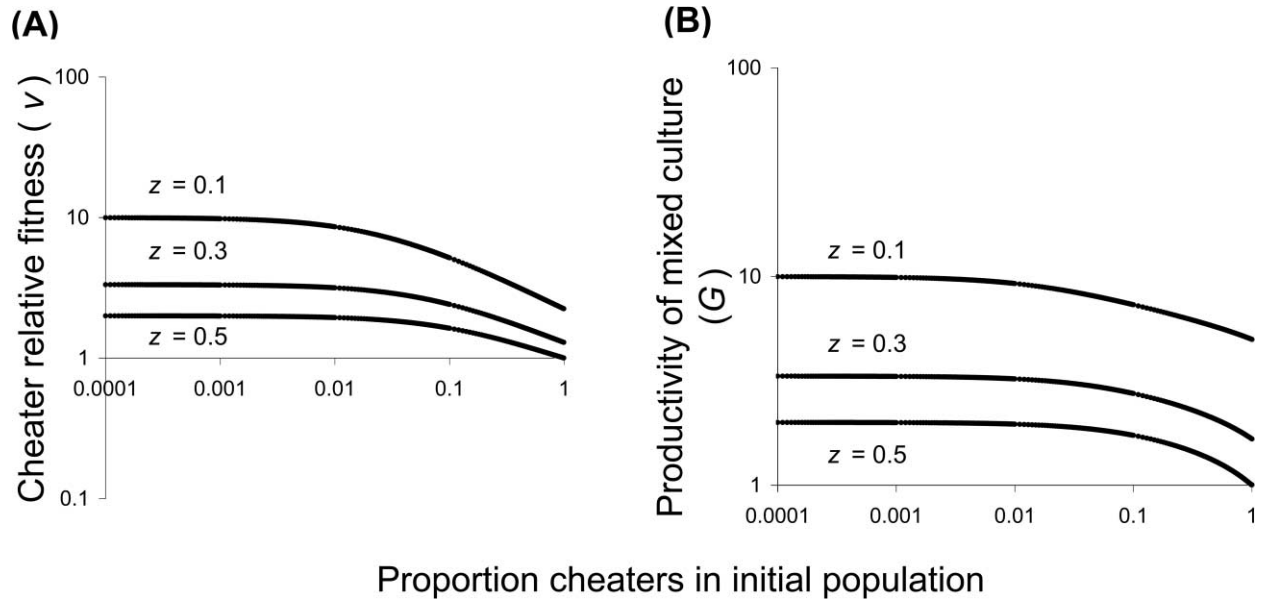


Figure 2: Logistic growth model, with more cooperative populations experiencing higher carrying capacity, for a range of initial population sizes ($z_0 = n_{c,0} + n_{d,0}$ as a proportion of carrying capacity for a fully cooperative population) and initial proportion of cheaters ($p_0 = n_{d,0}/z_0$). We assume that cheaters grow at twice the rate of cooperators and that they exert twice as great a competitive strain on resources ($a = b = 1$). A, Relative fitness of cheaters (v) decreases as initial population size (z_0) and initial proportion of cheaters (p_0) increases, because both result in reduced population growth and, hence, less pronounced fitness differences. B, Total productivity of the population (G) decreases as initial population size (z_0) and initial proportion of cheaters (p_0) increases, because both result in reduced population growth.

Hence, the relative fitness of cheaters versus cooperators is

$$v = e^{bT}. \quad (13)$$

It is easy to show that relative fitness of cheaters is frequency dependent and, indeed, that it is a decreasing function of cheater frequency:

$$\frac{dv}{dp_0} = \frac{\partial v}{\partial T} \frac{dT}{dp_0} = be^{bT} \frac{dT}{dp_0} \quad (14)$$

is negative because $dT/dp_0 < 0$. Note that frequency independence is recovered in the limit of weak selection (small b). Here, we can write $T = \hat{T} + O(b)$, where \hat{T} is a constant with respect to the initial frequency of cheaters, p_0 . Hence, from a Taylor expansion of equation (13), relative fitness is given by $v = 1 + \hat{T}b + O(b^2)$; that is, to a first-order approximation, it is independent of the frequency of cheaters.

This model of exponential growth, with an abrupt halt at a time depending on the population level of cooperation, is unrealistic and pursued only for the sake of analytic tractability. We now consider a more realistic model describing logistic growth and competition for resources, in which a population of cooperators can maintain a higher

cell density than can a population of cheaters. In particular, we continue to assume an intrinsic growth rate of 1 and $1 + b$ for cooperators and cheaters, respectively, but also include a density-dependence term in the dynamical equations describing growth:

$$\frac{dn_C}{dt} = n_{C,i}(1 - E_i), \quad (15)$$

$$\frac{dn_D}{dt} = (1 + b)n_{D,i}(1 - E_i), \quad (16)$$

where the numbers of cooperators and cheaters ($n_{C,i}$ and $n_{D,i}$) are expressed as proportions of the total population size that can be maintained at equilibrium (carrying capacity) if all cells cooperate and $E_i = n_{C,i} + (1 + a)n_{D,i}$ is the “effective” population size, in terms of the strain on resources that slows population growth, where each cheating individual incurs a strain that is equivalent to that of $1 + a$ cooperators. This reflects the benefit of cooperation for the population: for $a > 0$, cheaters inflate the effective size of the population so that, when they are common, the actual number of cells that can be sustained at stationary phase is reduced. This model contains nonlinearities that prevent an analytical treatment, although numerical solutions are possible (fig. 2), and these recover

the same qualitative results derived above for the simpler exponential growth model.

Methods

We experimentally tested our prediction that cheater fitness will be negatively correlated with the frequency of cheaters when population growth increases with cooperator frequency. We minimized the possible effects of structured populations leading to frequency dependence by carrying out growth in a shaken liquid media.

Description of Strains

Three *Pseudomonas aeruginosa* cooperator-cheater pairs (A–C) were used in this experiment. Each pair comprised a cooperator strain (“+”), a wild-type isolate that did produce pyoverdine (pvd), and a cheater strain (“–”), a pvd-defective mutant derived from the cooperator strain, giving A+, A–, B+, B–, C+, and C–. Specifically, the strains used were as follows. A+: PA01 (strain ATCC15692), a pvd-producing wild type; A–: PA06609 (Hohnadel et al. 1986), a pvd-negative mutant derived by ultraviolet mutagenesis from methionine auxotroph PA06409 (Rella et al. 1985), which in turn was generated by transposon mutagenesis from PA01; B+: PA01 (strain ATCC15692), a pvd-producing wild type, as for A+ above; B–: PA2399, an unmarked deletion mutant derived from PA01, defective for the pyoverdine synthetase gene *pvdD* (Ghysels et al. 2004); C+: a wild type, pvd-producing clone cultured from UCBPP-PA14, a clinical isolate known to also be pathogenic in plants and animals (Rahme et al. 1995); C–: a pvd-negative mutant coevolved with C+ in the laboratory under iron-limiting conditions, which should favor the spread of spontaneously arising mutants that somehow avoid the cost of producing siderophores and instead take up those produced by neighboring bacteria. Both C+ and C– were isolated from the same overnight culture, which at that point had been passaged through 19 (daily) serial transfers, where each transfer entailed the inoculation of 60 μ L of overnight culture (incubated at 37°C and shaken at 200 rpm) into 30-mL glass universal vials containing 6 mL fresh medium (CAA; 5 g casamino acids, 1.18g $K_2HPO_4 \cdot 3H_2O$, 0.25g $MgSO_4 \cdot 7H_2O$ L⁻¹). Like A– and B–, which are known to be pvd-synthesis defective, mature C– colonies also have a pale white appearance, distinguishing them from both C+ and their parent strain, which appear conspicuously green owing to the presence of pyoverdine.

Experimental Design

For each strain pair, “+” and “–” cultures were initiated from freezer stock and incubated overnight in an orbital shaker (37°C, 200 rpm). For this first stage, strains were grown in 6 mL standard King’s B (KB) medium in 30-mL glass universal vials. After 24 h, the cultures were retrieved and vortexed for 45 s. For pair A only, pilot data predicted substantial cell density differences between the A+ and A– cultures after 24 h growth in KB, so to equalize cell densities in the “+” and “–” cultures, a volume of A+ culture (3.64 mL) was drawn off and replaced with buffer solution (M9).

By appropriate dilution, we then prepared “treatment” cultures, mixing “–” (cheaters) with “+” (cooperators) at cell density ratios of approximately 1 : 1,000, 1 : 100, 1 : 10, 1 : 1, and 100 : 1. These cultures were supplemented with buffer (M9) such that each would contain cells at approximately the same density ($\sim 10^6$ cells/60 μ L). For each of these five treatments, we inoculated six replicate “competition” vials with 60 μ L of the prepared mixed cultures: 30-mL universal glass vials containing 6 mL CAA medium, as described previously, supplemented with 100 mg mL⁻¹ human apo-transferrin (an iron chelator) and 20 mM NaHCO₃, necessary for effective chelator activity (Meyer et al. 1996). The 30 competition vials were then placed in random order in an orbital shaker for overnight incubation (37°C, 200 rpm).

To assess the initial ratios of cooperators to cheaters in the inocula, multiple samples were taken and grown on KB-agar plates, and colony-forming units (CFUs) were counted and categorized as either “+” or “–” on the basis of color and morphology. Similarly, each of the 30 competition cultures, after 24 h of incubation at 37°C, was diluted, spread onto KB-agar plates, and incubated again overnight for counting. In this way, we obtained data on the absolute density and relative proportions of “+” and “–” CFUs both before and after the competition period. At each stage, labeling, spreading, and counting of plates were fully randomized to minimize order effects.

For pairs A and B, a single round of competition was conducted. In the case of pair C, two rounds were conducted, in each of which a different treatment failed to yield useful data. The results of both rounds were thus pooled for combined analysis.

We also carried out a separate experiment to test whether reducing the period of population growth reduced the extent of frequency dependence. Using strain pair A, we simultaneously inoculated two parallel sets of three treatments, with cheater : cooperator ratios of approximately 1 : 1,000, 1 : 10, and 100 : 1. Our protocol was identical to that described above except that, whereas one set

was cultured for a full 24 h before sampling, the second set was sampled after just 6 h of incubation.

Statistical Analyses

We performed two types of analyses on data from each of the three strain pairs. First, we calculated relative cheater fitness (v), by comparing the frequency of cheaters at the beginning and end of the experiment. Specifically, v is given by $v = x_2(1 - x_1)/x_1(1 - x_2)$, where x_1 is the initial proportion of cheaters in the population and x_2 is their final proportion. The value of v , therefore, signifies whether cheaters increase in frequency ($v > 1$), decrease in frequency ($v < 1$), or remain at the same frequency ($v = 1$). We assessed the nature and strength of the relationship between v and x_1 , using standard general linear models (GLMs), with x_1 as the explanatory variable. In each case, v and x_1 were first log transformed or, in the case of pairs 1 and 2, converted to the form $\log(v + 1)$ to normalize the distribution of the residuals, in accordance with the assumptions of parametric analyses. In the case of pair C, “round” was included as a blocking factor in the maximal model but was nonsignificant and so was excluded from the final model. Second, we quantified productivity (G) as the final cell density attained by the mixed culture at the end of the competition period. Again, this was analyzed with respect to initial cheater frequency, x_1 , using a standard GLM. Here again, G was first log transformed to normalize the distribution of the residuals. All analyses were performed with Minitab 14.1 (<http://www.minitab.com>).

Results

In all three cheater-cooperator pairs, the relative fitness of cheaters was significantly negatively correlated with initial cheater frequency (fig. 3A). In all cases, the relationship was best described by a power function (pair A: $v = 4.200x_1^{-0.442}$, GLM: $F = 92.17$, $df = 1, 28$, $P < .001$; pair B: $v = 1.856x_1^{-0.123}$, GLM: $F = 11.27$, $df = 1, 26$, $P = .0024$; pair C: $v = 2.270x_1^{-0.176}$, GLM: $F = 37.24$, $df = 1, 45$, $P < .001$). At low frequencies, cheaters showed higher fitness than cooperators, while at high frequencies, cheater fitness was comparable to—or in the case of pair A, significantly lower than—that of cooperators.

For all three strain pairs, productivity of the mixed culture was significantly negatively correlated with initial cheater frequency (fig. 3B). In all cases, the relationship was best described by an exponential function (pair A: $G = 3.56 \times 10^8 e^{-1.97x_1}$, GLM: $F = 74.83$, $df = 1, 28$, $P < .001$; pair B: $G = 2.82 \times 10^8 e^{-2.38x_1}$, GLM: $F = 75.82$, $df = 1, 26$, $P < .001$, $r^2 = 0.70$; pair C: $G = 1.04 \times 10^9 e^{-2.13x_1}$, GLM: $F = 139.11$, $df = 1, 51$, $P < .001$). In cultures initi-

ated with a high frequency of cheaters (i.e., the 100 : 1 treatment), productivity was around an order of magnitude lower than in cultures where cheaters were scarce (i.e., the 1 : 1,000 treatment).

The strength of frequency dependence was reduced when populations were allowed to grow for shorter periods of time (fig. 4). In our further experiment with strain pair A, the population growth was significantly lower in the 6-h-old cultures than in the 24-h-old cultures (fig. 4B; 24-h cultures: $G = 1.01 \times 10^8 e^{-2.779x_1}$, 6-h cultures: $G = 1.68 \times 10^6 e^{3.824x_1}$; GLM: final density \times time $F = 119.76$, $df = 1, 29$, $P < .001$). The strength of frequency dependence with respect to cheater relative fitness was also reduced over the shorter time period (fig. 4A; 24-h cultures: $v = 1.303x_1^{-0.241}$, 6-h cultures: $v = 1.117x_1^{-0.036}$; GLM: fitness \times time $F = 13.06$, $df = 1, 27$, $P < .001$).

We carried out a number of additional analyses to test the robustness of our results. First, we considered an alternative measure of productivity: the fold increase in total cell density over the competition period. These analyses produced results qualitatively concordant with those presented here. Second, we considered an alternative approach for all fitness versus proportion analyses: instead of regressing x_1 against v (an expression that itself contains x_1), we simply regressed the initial odds ratio, $x_1/(1 - x_1)$ against the final odds ratio, $x_2/(1 - x_2)$. By this approach, fitted regression lines with slopes significantly less than 1 indicate negative frequency dependence. In the first set of experiments, we found this result for all three strain pairs (pair A: slope 0.73 ± 0.02 , $T_s = 12.30$, $df = 28$, $P < .001$; pair B: slope 0.93 ± 0.03 , $T_s = 2.32$, $df = 26$, $P = .029$; pair C: slope 0.84 ± 0.04 , $T_s = 4.33$, $df = 48$, $P < .001$). In the experiment considering different competition periods, only the 24-h cultures showed negative frequency dependence (24-h cultures: slope 0.84 ± 0.02 , $T_s = 7.68$, $df = 16$, $P < .001$; 6-h cultures: slope 0.97 ± 0.03 , $T_s = 1.29$, $df = 14$, $P = .218$). Third, we repeated all analyses using the ratio of cheaters to cooperators (x_R) as the explanatory variable, rather than the proportion of cheaters in the mixed population (x_1), where $x_R = x_1/(1 - x_1)$. For all three strain pairs, results obtained in regressions with x_R were qualitatively analogous to those presented here.

Discussion

In this study, we have determined under what conditions the production of public goods in microbes will be subject to frequency-dependent selection. Specifically, we have shown that if there is strong selection, two sets of conditions can lead to frequency-dependent fitness: (1) when cooperators acquire more than a random benefit of public good production—for example, if cooperators tend to be

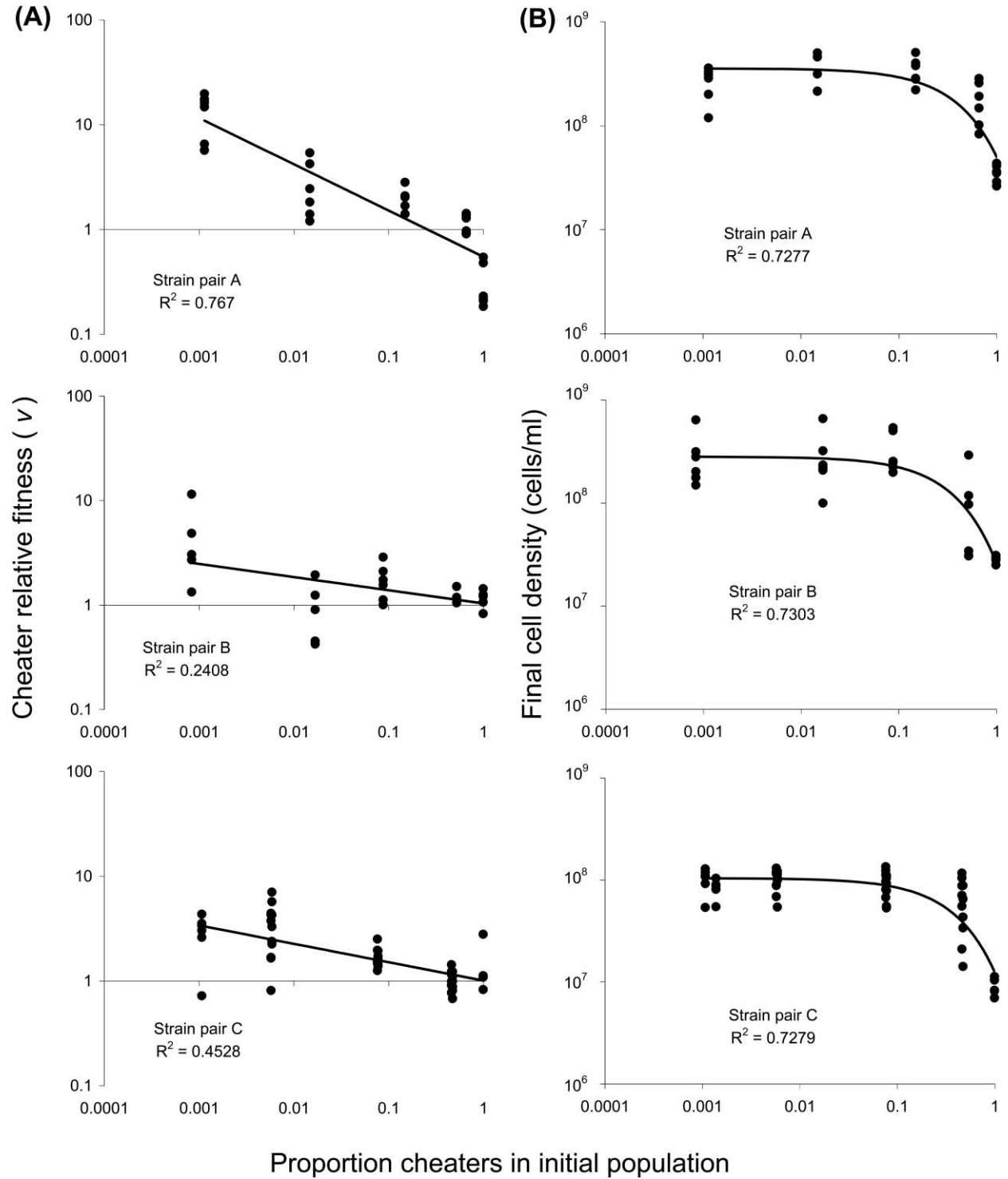


Figure 3: Relative cheater fitness (A) and mixed-culture productivity (B) as functions of initial cheater frequency for three independent pairs of *Pseudomonas aeruginosa* strains. Here, relative fitness is the proportional increase in frequency of cheaters relative to cooperators, and productivity is the final cell density of the mixed culture after a 24-h competition period. Fitted lines reflect power (A) and exponential regression (B) curves estimated by least squares.

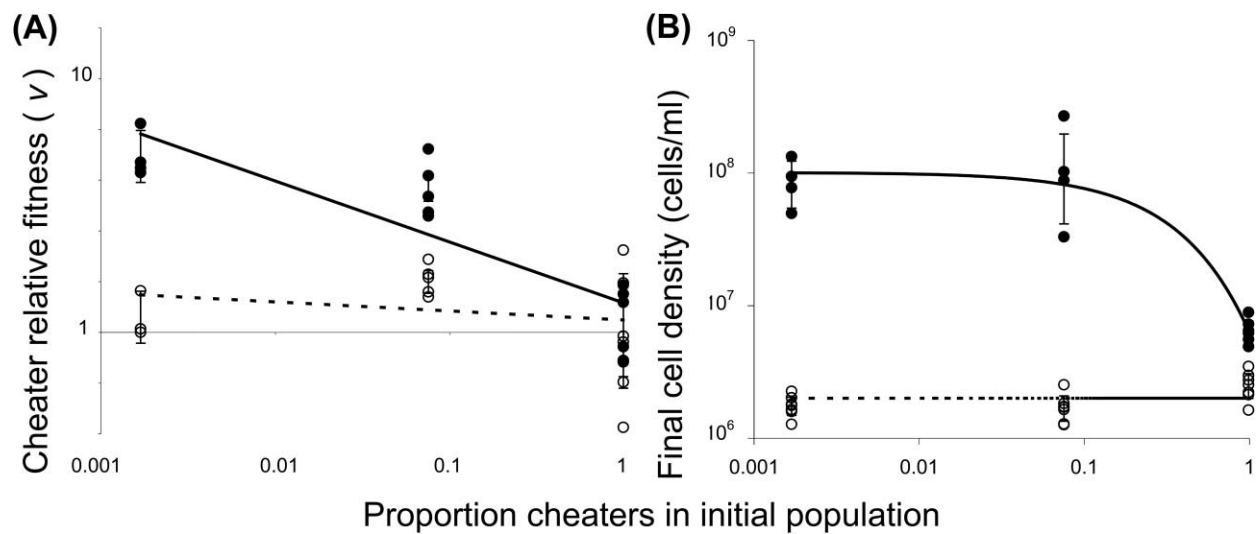


Figure 4: Relative cheater fitness (A) and mixed-culture productivity (B) as functions of initial cheater frequency in 6-h cultures (open circles, dotted regression line) versus 24-h cultures (solid circles, solid regression line). Here, relative fitness is the proportional increase in frequency of cheaters relative to cooperators, and productivity is the final cell density of the mixed culture at the end of the competition period. Fitted lines reflect power (A) and exponential (B) regression curves estimated by least squares. Error bars reflect 95% confidence intervals around the means.

clustered together (population structuring) or if individual cells have preferential access to the public goods that they produce (fig. 1); or (2) when increased levels of cooperation lead to greater growth, a higher carrying capacity, and hence more generations over which cheaters can exploit cooperators (fig. 2). In both cases, the relative fitness of cooperators or cheaters is greater when they are less common. We then explicitly tested the second of these possibilities (by controlling for the first, population structuring), using the production of iron-scavenging siderophore molecules in *Pseudomonas aeruginosa* as a model trait. As predicted, we found a negative relationship between the relative fitness of cheaters and the proportion of cheaters in the population (fig. 3A). Furthermore, we found support for the underlying assumption leading to this prediction—a higher proportion of cooperation led to greater growth (fig. 3B), and a reduction in the period of growth led to a decrease in the extent of frequency dependence (fig. 4).

Situations Favoring Frequency-Dependent Selection

We have identified two ways in which kin selection models of public goods dilemmas can generate frequency-dependent selection, given strong selection. First, population structure, incorporating both limited mixing of cells and limited diffusion of public goods molecules (local social interaction) can result in the selective advantage of cheating decreasing as cheaters become more common. When

populations are structured, the level of public goods available to an individual will depend on (1) the production of public goods by that individual and its clonal relatives and (2) the average level of public goods production in the population. Increasing population structure increases the importance of the former and decreases the importance of the latter. A higher frequency of cheaters will lead to a lower average level of public goods production (2). The consequences of this will be greater for cheaters, because they receive fewer public goods through their own production and that of their relatives (a). This leads to the relative fitness of cheaters decreasing with cheater frequency. Second, frequency dependence may also arise when total population growth increases with the level of cooperation in the population. If more cooperative populations achieve a higher carrying capacity, then more rounds of growth and division are possible before stationary phase is reached. The selective benefit of cheating multiplies with each round of division, so that the relative fitness of a cheater measured over the period of population expansion increases with the (initial) frequency of cooperators in the population (fig. 2A).

How does our work relate to previous theory? We have provided some specific cases for the general conclusion that the generation of frequency-dependent selection relies on strong selection (Charlesworth 1978; Michod 1982; Toro et al. 1982; Queller 1984; Nowak and May 1992; Day and Taylor 1998; Rousset 2004; Jansen and Van Baalen 2006). The aim of this previous work was to elucidate general

points, and so scenarios were modeled that do not facilitate simple application to specific systems such as public goods production (e.g., two-player games; Queller 1984). An important exception to this was provided by Dugatkin et al. (2003), who explicitly considered frequency-dependent selection in microbes. However, Dugatkin et al. (2003) did not explain how frequency dependence should occur in the first place—their aim was to address the consequences of frequency-dependent selection, and so they simply assumed that it occurred. This is analogous to taking equation (7) or (13) as a starting point rather than determining how they should arise. More generally, frequency dependence does not usually arise from most of social evolution theory because selection is usually assumed to be weak, to make analyses more tractable (Hamilton 1964; Taylor and Frank 1996; Frank 1998; Rousset 2004). Frequency dependence can also arise in models for the evolution of cooperation that do not rely on kin selection (e.g., reciprocity; Axelrod and Hamilton 1981).

Frequency-Dependent Selection in Microbes

Frequency-dependent selection can, under certain conditions, prevent cheater takeover and facilitate the coexistence of multiple phenotypes (Aviles 2002). However, in two of the three strain pairs examined in this study, cheaters were at least as fit as cooperators, even when common (fig. 3A). With such a payoff structure, we may anticipate that, in time, cheaters would go to fixation in populations where there is little competition between groups. Consistent with this idea, siderophore-defective strains are routinely isolated from the lungs of late-stage cystic fibrosis patients, where they typically grow as monoclonal populations (Lee et al. 2005; Salunkhe et al. 2005; Smith et al. 2006). On the other hand, in an environment where competition occurs between subpopulations and these subpopulations contain different proportions of cheaters, those groups with more cooperators may outcompete cheater-dominated groups (Griffin et al. 2004). Thus, cooperation may also be maintained by frequency-dependent selection at the level of the group.

We suggest that negative frequency dependence should be common in microbes, because (1) both population structuring and cooperation-dependent growth rates are likely to be important in many situations, and (2) mutations can occur that lead to large differences in the level of cooperation (strong selection; see below). Accordingly, negative frequency-dependent selection has now been shown for social cheaters in at least 12 studies on seven microbial species (Velicer et al. 2000; Vulic and Kolter 2001; Fiegna and Velicer 2003; Rainey and Rainey 2003; Turner and Chao 2003; Dugatkin et al. 2005; Brockhurst et al. 2006; Harrison et al. 2006; MacLean and Gudelj 2006;

Ellis et al. 2007; Gilbert et al. 2007; this study). However, while we highlight these studies as support for our prediction, we also point out that most did not explicitly test the underlying assumptions of why frequency dependence occurs. Ultimately, a range of complementary approaches is most useful. At one extreme will be studies designed to reveal those specific mechanisms responsible for frequency dependence. For example, our aim in this study was to test one particular mechanism that can lead to frequency dependence—cooperation-dependent population growth. In order to do this, we (1) carried out our experiment in shaken vials, in order to remove the other possible mechanism that can lead to frequency-dependent selection—population structure—and (2) tested the underlying assumption that a higher proportion of cooperators allows the population to grow to a larger size (fig. 3B). At the other extreme are studies in more natural conditions that examine the net consequences of both mechanisms for generating frequency dependence (e.g., Harrison et al. 2006). An important task for the future is to determine the relative importance in nature of the two different mechanisms that we have shown can lead to frequency dependence.

We observed a decrease in the extent of frequency dependence when the period of time available for growth was shortened (fig. 4). This supported the assumption of our model that the relative fitness of cheats increases over time as the population goes through more rounds of growth and division. It is possible that other factors could also cause the relative fitness of cheaters to vary over time. For example, as the population grows, this could lead to a decrease in iron availability that reduces the relative fitness of cheaters (Griffin et al. 2004) or a higher population density that allows cheaters to better exploit cooperators and hence increases their own relative fitness (Greig and Travisano 2004; MacLean and Gudelj 2006). However, effects such as these cannot explain the frequency dependence evident in our data; in order for frequency dependence to be observed, one of the additional factors that we have suggested would still be required, such as populations with greater frequencies of cooperators growing to higher final densities.

More generally, our study illustrates that, although the same social evolution theory originally developed for metazoans can be applied to microbes, certain aspects of microbial biology mean that some differences may occur in the evolution of social traits. In particular, with typical social evolution study organisms, such as insects, birds, and mammals, it is usually assumed that mutation will lead to minor variations in behavior (weak selection), and so frequency dependence will be relatively unimportant (Sinervo and Lively 1996; Sinervo et al. 2006; Sinervo and Calsbeek 2006). In contrast, in the case of microbes, single

or small numbers of mutations can have extremely large effects on social behaviors (strong selection; Velicer et al. 1998, 2000; Rainey and Rainey 2003; Velicer and Yu 2003; Foster et al. 2004; Griffin et al. 2004; Fiegna et al. 2006). For example, in *P. aeruginosa*, social behaviors such as siderophore production are routinely and completely lost from isolates obtained from lung infections (Lee et al. 2005; Salunkhe et al. 2005; Smith et al. 2006). Furthermore, the majority of microbial cooperative behaviors are controlled by quorum sensing regulatory networks (Keller and Surette 2006; Venturi 2006; Diggle et al. 2007). Consequently, the disruption of these networks, even through the loss of a single gene, can have huge effects on the level of cooperation. A consequence of the potential for mutations of large effects (strong selection) in microbes, is that this allows frequency-dependent selection, through the mechanisms we have described in this study.

Acknowledgments

We thank A. Buckling and P. Cornelis for providing bacterial strains, B. Evans for laboratory assistance, and N. Colegrave, R. Kümmerli, G. Wild, and an anonymous reviewer for discussion and comments on the manuscript.

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Associate Editor: Thomas N. Sherratt
Editor: Michael C. Whitlock

VIRAL EPIZOOTIC REVEALS INBREEDING DEPRESSION IN A HABITUALLY INBREEDING MAMMAL

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Received January 29, 2007

Accepted May 8, 2007

Inbreeding is typically detrimental to fitness. However, some animal populations are reported to inbreed without incurring inbreeding depression, ostensibly due to past “purging” of deleterious alleles. Challenging this is the position that purging can, at best, only adapt a population to a particular environment; novel selective regimes will always uncover additional inbreeding load. We consider this in a prominent test case: the eusocial naked mole-rat (*Heterocephalus glaber*), one of the most inbred of all free-living mammals. We investigated factors affecting mortality in a population of naked mole-rats struck by a spontaneous, lethal coronavirus outbreak. In a multivariate model, inbreeding coefficient strongly predicted mortality, with closely inbred mole-rats ($F \geq 0.25$) over 300% more likely to die than their outbred counterparts. We demonstrate that, contrary to common assertions, strong inbreeding depression is evident in this species. Our results suggest that loss of genetic diversity through inbreeding may render populations vulnerable to local extinction from emerging infectious diseases even when other inbreeding depression symptoms are absent.

KEY WORDS: Coronavirus, disease susceptibility, *Heterocephalus glaber*, inbreeding depression, purging.

Inbreeding depression, the negative fitness effects of mating among relatives, has been well documented in laboratory, agricultural, and natural settings (e.g., Charlesworth and Charlesworth 1987; Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Keller and Waller 2002). Nonetheless, great variance exists in the magnitude of inbreeding depression within and among populations (e.g., Pray and Goodnight 1995; Lacy and Ballou 1998; Haag et al. 2003) and some populations, despite high levels of inbreeding, show little or no evidence of inbreeding depression (e.g., Keane et al. 1986; Doums et al. 1996; Kalinowski et al. 1999; Visscher et al. 2001; Duarte et al. 2003; Bilde et al. 2005; Peer and Taborsky 2005; Milot et al. 2007). Such a lack of inbreeding depression is often attributed to “purging,” the removal

of deleterious alleles by strong selection, during historical periods of extended inbreeding (Lande and Schemske 1985; Barrett and Charlesworth 1991). Although there is empirical support for reduced inbreeding depression with continued inbreeding (e.g., Barrett and Charlesworth 1991; McCall et al. 1994; Crnokrak and Barrett 2002), recent studies, both theoretical (Wang et al. 1999; Wang 2000; Whitlock 2002; Glémin 2003) and empirical (Barrett and Charlesworth 1991; Ballou 1997; Lacy and Ballou 1998; Byers and Waller 1999; Crnokrak and Barrett 2002; Boakes et al. 2007), have demonstrated that purging is not always effective. Alleles that are only deleterious under certain environmental conditions, for example, will only be disfavored by natural selection when those particular conditions arise (Bijlsma et al. 1999). Even habitual inbreeders would thus be expected to still harbor alleles that would be deleterious under some different, novel selection regime.

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The eusocial naked mole-rat, *Heterocephalus glaber*, is a classic example of a habitual inbreeder seemingly impervious to inbreeding depression; “the only mammal species that has been shown to undergo continuous close inbreeding with no obvious effects of inbreeding depression” (Bromham and Harvey 1996). An early microsatellite study of wild colonies suggested that over 80% of all mating occurs between first-degree relatives (Reeve et al. 1990)—an unprecedented level of inbreeding among vertebrates. The lack of inbreeding avoidance and the naked mole-rats’ intense xenophobia (Lacey and Sherman 1991) have been described as “mechanisms that apparently minimize the chance of ever outbreeding” (Jarvis et al. 1994). More recently, however, some circumstantial evidence has emerged of some latent selection for outbreeding: O’Riain et al. (1996) and Braude (2000) demonstrated the existence of rare dispersal morphs, and Clarke and Faulkes (1999) and Ciszek (2000) demonstrated a weak mating preference for nonkin. However, in 25 years of intensive study no overt signs of inbreeding depression have been reported. This makes the naked mole-rat a particularly interesting case to test the hypothesis that novel selection regimes may uncover inbreeding depression.

In this study, we performed a retrospective investigation of the effects of inbreeding on naked mole-rat mortality through a spontaneous outbreak of a novel viral pathogen. In doing so, we uncovered the first evidence of a substantial cost to continuous close inbreeding in this species.

Methods

STUDY POPULATION

Our population, initiated with wild-caught founders from various localities in Kenya, has been maintained since 1981 in custom-built facilities at the University of Cape Town. Husbandry details have been described previously by Jarvis (1991). Colonies typically comprised a single breeding pair and up to 55 nonbreeding relatives of both sexes. In total, our sample comprised 365 animals in 10 complete colonies: 209 males and 159 females, including 11 and 10 breeders of either sex, respectively. Age and body mass were known for all animals (age: 5 to 273 months, mean 89.4 ± 60.4 SD; mass: 11.9 to 83.2 g, mean 35.7 ± 12.7 SD). Furthermore, for each individual, a single dam and sire could be identified with certainty, on the basis of their distinctive morphologies and behavioral profiles (Clarke and Faulkes 1998). Based on these records, we constructed a pedigree for each individual (maximum length: five generations) and used it to estimate a coefficient of inbreeding (F) as per Wright (1922). Our study violates a key assumption of Wright’s approach, however: that pedigree founders are unrelated. Population genetic studies (Faulkes et al. 1990; Reeve et al. 1990) suggest that intracolony relatedness is typically very high in wild naked mole-rats. Thus, here we explicitly assumed

that pedigree founders captured from the same exact locality (i.e., same colony) were siblings. Parallel analyses in which founders were held to be unrelated yielded qualitatively concordant analyses (results not shown). Inbreeding coefficients ranged from $F = 0$ (outbred) through to $F = 0.5$ (highly inbred) with mean $F = 0.163 \pm 0.158$ SD.

DISEASE OUTBREAK

In September 2002, an emergent strain of virulent enteric coronavirus swept unchecked through our captive naked mole-rat study population, causing acute diarrhea, dehydration, and severe enteric hemorrhaging. The day of first exposure in each colony was determined from the first appearance of symptoms (dry, unused toilet chamber), and in each case, the first deaths were recorded within two days. Coronaviruses spread easily by faeco-oral or aerosol transmission (Weiss and Navas-Martin 2005), so we assumed that all individuals in a colony were exposed to the virus simultaneously. No attempts were made to medicate infected animals. Mortality was monitored daily and dead animals were removed immediately for identification and confirmation of the presence of disease symptoms.

STATISTICAL ANALYSIS

To identify factors affecting survival, we used proportional hazards regressions (Therneau and Grambsch 2002), implemented with the PHREG procedure (with the TIES = exact option), in SAS (SAS Institute, Cary, NC). Our data conformed to the assumption of proportional hazards; no evidence of nonproportionality was apparent in the smoothed, scaled Schoenfeld residual plots (Therneau and Grambsch 2002) and the inclusion of a time-dependent covariate had a nonsignificant effect ($P = 0.09$). Model selection followed the “main-effects first” model-building strategy of Hosmer and Lemeshow (2000). Only the effects shown in Table 1 were entered into the final model; we excluded body mass as an explanatory variable after preliminary runs showed that its presence did not significantly improve the model (likelihood ratio test: $\chi^2 = 1.27$; $P = 0.26$). All two- and three-way interaction terms not shown in Table 1 were excluded in a similar fashion. Because the 10 colonies were housed in three separate constant environment rooms, we accounted for possible location effects by stratifying the final model by “room number,” and nested within that, “colony.” An unstratified model, however, yielded virtually identical results. To assess the relative effect of different levels of inbreeding on survival, we calculated hazard ratios (Therneau and Grambsch 2002) for F at discrete values. Finally, inbreeding depression was estimated in terms of the number of lethal equivalents per gamete, calculated as per Kalinowski and Hedrick (1998).

Table 1. Factors affecting the probability of death during the coronavirus epizootic.

Variable	df	Parameter estimate	SE	χ^2	<i>P</i>	Hazard ratio	95% confidence limits for hazard ratio	
Inbreeding <i>F</i>	1	4.422	1.444	9.379	0.0022	83.295	4.914	1411.805
Age (months)	1	0.006	0.002	8.008	0.0047	1.006	1.002	1.011
Sex	1	0.362	0.179	4.078	0.0434	1.436	1.011	2.041
Breeding status	1	1.012	1.383	0.536	0.4642	2.752	0.183	41.399
Age \times Breeding status	1	−0.017	0.006	7.677	0.0056	0.983	0.971	0.995
Sex \times Breeding status	1	2.821	1.096	6.624	0.0101	16.789	1.959	143.863

Results

In just eight weeks, acute diarrhea, dehydration, and severe enteric haemorrhaging associated with the coronavirus killed 161 of 365 animals (44.1%). Survival was significantly lower among more inbred animals (Fig. 1). Statistically controlling for the effects of other explanatory variables (Table 1), we found that offspring produced by half-sibling ($F = 0.125$) and full-sibling ($F = 0.250$) parent pairs were, respectively, 174% and 302% more likely to die than the offspring of unrelated parents ($F = 0$). Inbreeding depression in survival, measured as lethal equivalents, was calculated as $B = 1.13$ (95% CI: 0.50–1.81).

Age was also highly significantly correlated with susceptibility, with 10 years of age increasing mortality risk by 212.5% among nonbreeders (Fig. 2). Sex too was a significant factor, with females (143.6%) more likely to survive than males. Breeding status interacted significantly with sex. Queens had high survival despite their advanced age (eight of 10 survived), whereas only two of 11 male breeders survived.

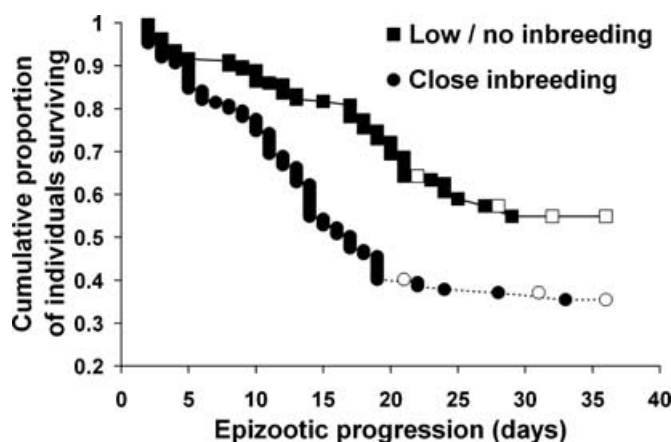


Figure 1. Kaplan–Meier plots showing the proportional survival of highly inbred ($F \geq 0.25$) versus outbred and mildly inbred naked mole-rats ($0 \leq F \leq 0.125$) through the course of the coronavirus outbreak. Open markers denote censored datapoints. Although represented categorically here, inbreeding was treated as a continuous variable for all regression analyses.

Discussion

This study provides the first clear evidence of a substantial cost to inbreeding in this habitual inbreeder. Inbred naked mole-rats were significantly more likely to die than their outbred counterparts, displaying inbreeding depression of a magnitude similar to that observed in other captive rodents (naked mole-rats: $B = 1.13$; other rodents: mean $B = 1.15$; calculated for $n = 7$ captive taxa; Ralls et al. 1988). Note, however, that this estimate of lethal equivalents does not account for variation in other variables (sex, age, breeding status) that were found to affect mortality (Table 1), and may thus be an underestimate. Moreover, this figure does not account for Allee effects associated with the obligate social lifestyle of naked mole-rats. In the wild, naked mole-rat colonies persist under harsh environmental conditions because the cooperative efforts of multiple foragers enable them to exploit patchy food resources. The loss of 87% of the workforce (as we observed in one colony) would almost certainly lead to local extinction of a wild colony. Thus, the true costs of inbreeding in the wild may well be higher than the estimates presented here.

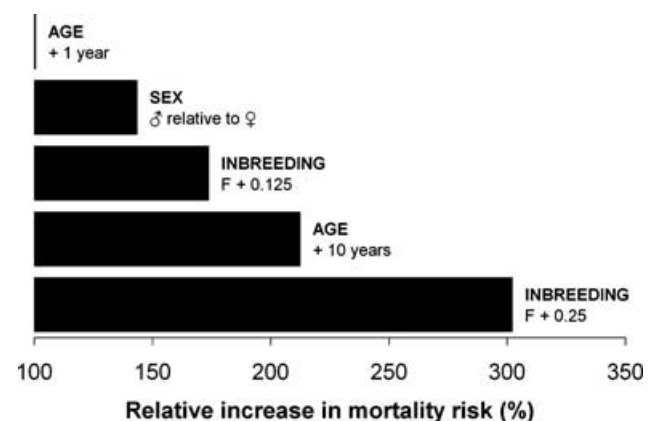


Figure 2. Comparison of selected variables' effects on the risk of death during the coronavirus epizootic. These values are derived from hazard ratio estimates generated by the proportional hazards model and reflect the change in likelihood of death following a given change of a predictor variable from its base condition (i.e., 0 for continuous variables, females in the case of sex). Age and sex effect estimates shown here apply to nonbreeders only.

Mechanistically, inbreeding increases homozygosity, leaving inbred individuals with lower genetic diversity than their outbred counterparts. There is much interest in the relationship between host genetic diversity (at key loci such as the major histocompatibility complex, as well as more generally in the genome) and resistance to parasites and disease (O'Brien and Evermann 1988; Hamilton et al. 1990; Hedrick 1994). The results of this study augment mounting evidence from a broad range of taxa that reduced genetic diversity, as can arise through inbreeding, is associated with increased susceptibility to infection (O'Brien et al. 1985; Black 1992; Carrington et al. 1999; Coltman et al. 1999; Meagher 1999; Schmid-Hempel and Crozier 1999; Cassinello et al. 2001; Hedrick et al. 2001; Messaoudi et al. 2002; Acevedo-Whitehouse et al. 2003; McClelland et al. 2003; Reid et al. 2003; Spielman et al. 2004; Wegner et al. 2004; Hawley et al. 2005; Calleri et al. 2006; Whiteman et al. 2006; Luong et al. 2007; Reid et al. 2007; Seeley and Tarpy 2007).

Our secondary findings, that susceptibility increased with age and was higher in males, both conform to established theory. Immune responsiveness is known to decline with age in various taxa (Adamo et al. 2001; Linton and Dorshkind 2004), with a range of symptoms including a marked decline in T-cell production. Similarly, a male bias in susceptibility is a well-documented phenomenon (Klein 2000), and in vertebrates is hypothesized to result at least in part from the immunosuppressive burden of testosterone (Folstad and Karter 1992). Our data provide circumstantial support for this hypothesis, in that breeding males, which typically show highly elevated testosterone levels (Clarke and Faulkes 1998), were especially vulnerable to the virus. The high survival among queens was unexpected, but may also have been partially hormone driven: glucocorticoid stress hormones, also known to be immunosuppressive (Sapolsky 1992), are typically lower in naked mole-rat queens than in other colony members, except during pregnancy (Faulkes and Abbott 1997). At such times stress hormone levels are elevated, most likely in response to acute energetic stress (Råberg et al. 1998), and immune function is typically impaired (e.g., Nordling et al. 1998). Accordingly, of four mole-rat queens pregnant at the onset of the infection, two aborted and survived, whereas the two that carried their pups to full term succumbed to the virus. Other potential sources of energetic stress, and hence immunosuppression, such as differences in nutritional status (Gershwin et al. 1985), workload (Deerenberg et al. 1997), or thermoregulatory burden (Nelson and Demas 1996), are unlikely to have contributed substantially to these results because all animals had lifelong access to ad libitum food and lived in a finely regulated physical environment.

In this study, we have demonstrated that inbreeding is associated with increased susceptibility to a viral epidemic in a habitual close inbreeder, ostensibly free from other inbreeding depression effects. These results have important implications for our under-

standing of animal populations with reduced genetic variability. Inbred individuals may remain more vulnerable to emergent infectious diseases than their outbred counterparts, despite extensive purging of the background genetic load. Thus, assessment of inbreeding depression in the absence of severe disease outbreaks may dramatically underestimate the true fitness costs of inbreeding.

More generally, our results emphasize that purging cannot remove deleterious alleles unless these alleles are exposed to selection (Bijlsma et al. 1999). Alleles temporarily "hidden" from selection, such as those conferring susceptibility to novel or rare diseases, may accumulate over time without negatively affecting fitness. However, when an appropriate pathogen is ultimately encountered, these alleles may start to strongly affect fitness and thus will be exposed to selection. Our finding of increased mortality among inbred mole-rats during a coronavirus outbreak reflects such purging in action.

ACKNOWLEDGMENTS

We thank J. U. M. Jarvis for pedigree records and H. Kokko and two anonymous reviewers for helpful discussions. Financial support was provided to JOR by the South African National Research Foundation. This research adhered to the Animal Behavior Society Guidelines for the Use of Animals in Research, the legal requirements of the country in which the work was carried out, and all institutional guidelines.

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Associate Editor: H. Kokko

Quick guide

Meerkats

Adin Ross-Gillespie
and Ashleigh S. Griffin

What is a meerkat? Meerkats (*Suricata suricatta*) are small (<1 kg) mongooses found in arid regions of southern Africa. They feed mainly on insects, arachnids and small vertebrates, and spend much of their time digging in the sand in pursuit of their prey. Unlike many other small mammals in such environments, meerkats are strictly diurnal. By night, they shelter in elaborate burrow systems; at dawn they emerge to roam their territories, which may extend for several square kilometres and encompass dozens of overnight burrows and hundreds of emergency 'bolt-holes' that provide refuge from predators such as eagles or jackals.

What kind of a name is 'meerkat' anyway? Meerkatzen is the Germanic name for Cercopithecine monkeys, which in turn probably derives from the Sanskrit for monkey — markata. Presumably, to the explorers that first described meerkats, their appearance and/or behaviour was reminiscent of monkeys. So now you know.

Is Timon from Disney's *The Lion King* a fair representation of a meerkat? Not really... In the wild, a solitary meerkat is a rare and typically short-lived phenomenon; indeed, the single most striking thing about meerkats is that they are highly social animals. They live year-round in tight-knit groups of up to 40 individuals presided over by a dominant female and a dominant male who fathers most of the litters born in the group.

What's so special about meerkat sociality? Meerkats exhibit a rich repertoire of cooperative behaviours. Observe a group of meerkats at their burrow at the end of a day's foraging, and you'll

see piles of individuals huddling together for warmth and grooming one another. You may also see digging teams working together to maintain the tunnels of their burrow. By day, while the group forages, individuals take turns to stand guard atop nearby bushes or termite mounds. These sentries scan the sky and surroundings and signal to the group at the first sign of approaching danger. When rivals are encountered, meerkat groups band together to defend their territories, and the crucially important burrows they contain. At other times, they mob together to ward off larger predators, such as cobras. Cooperation is most striking, however, when it comes to rearing young. The matriarch produces most of the group's offspring, but raising these pups is a responsibility shared by all group members. For the first month, helpers babysit pups at the burrow, losing up to 11% of their body mass in the process. Older females may even suckle the young. Later, when the pups begin to forage with the group, helpers provision them with a large share of the prey that they manage to find.

Is life as a meerkat a Utopian idyll? In meerkats, as in our own society, cooperation is seldom to be found without its

ugly twin, conflict. Throughout the day, foragers frequently attempt to steal prey items from one another, but the most serious conflicts occur over who gets to breed. Males from rival groups, prospecting for receptive females, are often attacked by resident males, and dominant males jealously guard their mates during oestrus. Competition for dominance is fierce: a coup d'état is always a threat, and a regime of bullying is necessary to keep order in the ranks. If a subordinate female falls pregnant, she risks a brutal beating, or worse, expulsion from the safety of the group. Even if she does manage to stay until giving birth, her newborn pups may well be killed by the dominant female.

Why do meerkats cooperate? Social evolution theory would suggest that meerkat cooperation may largely be down to high levels of genetic relatedness: by helping to raise the offspring of relatives, individuals indirectly transmit copies of their own genes. In meerkats, however, this is not a complete explanation, because unrelated immigrants may help just as much as other group members. Research suggests that, in the harsh environment in which meerkats live, belonging to a group — the



Figure 1. Meerkats on the lookout.

larger the better — is absolutely vital. Without sentries, meerkats cannot feed safely; without helpers, they cannot breed successfully; without strength in numbers, they cannot hold territory. Through cooperation, individuals can increase the size and success of their group, and in so doing, improve their own individual prospects for survival and reproduction.

Do all meerkats help equally?

No, research has shown that the amount of help provided typically depends on the ability to help: well-fed individuals are more likely to expend energy for the benefit of others. But this is not to say that meerkats simply help indiscriminately. Female helpers, for example, preferentially feed female pups — probably because these pups, being the philopatric sex, represent the future workforce that might one day help to raise the helpers' own litters.

How is it that we know so much about meerkats? Meerkats' striking sociability and their tendency to become habituated to the presence of human observers, make them eminently tractable model organisms for investigations into the evolution of social behaviour. A large-scale, long-term field study of meerkats in the southern Kalahari was initiated in 1993 by Tim Clutton-Brock of the University of Cambridge. This project, involving hundreds of thousands of man-hours of detailed behavioural observations coupled with long-term pedigree and life-history information has provided a wealth of data on cooperative breeding behaviour and constitutes the best database of such information that exists today for social mammals.

What else have we learned from meerkats? Apart from serving as a model for the evolution of cooperation, the Kalahari meerkats have provided unique opportunities for research in other areas of biology too. Through observation and experiments, Marta Manser and her group at the University of Zurich have 'decoded'

many of the 30 or so calls, and the numerous postures, displays and olfactory signals that meerkats use. This work extends our understanding of animal communication and cognition. For example, meerkat alarm calls, which categorise threats according to both class and urgency, exhibit a level of complexity more typically associated with humans. Other behavioural studies have used meerkats to investigate why animals play, and recently they were used to neatly demonstrate that animals can, and do, actively teach their young. On the ecological front, meanwhile, meerkats have provided insight into the demographics, dispersal, and population genetics of social carnivores. For instance, long-term population data clearly demonstrate the "Allee effect": if meerkat group sizes fall too low, recruitment tails off rapidly and local population crashes can ensue. Finally, at a physiological level, meerkats are helping us to better understand the energetics, endocrinology and thermoregulation of small desert-adapted mammals.

Where can I learn more about meerkats?

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Primer

Structure and function of the cerebral cortex

Stewart Shipp

The grey matter of the cerebral cortex is a convoluted, layered sheet of tissue, 2–3 millimetres thick in man but with a surface area of several hundred square centimetres. This is not an adaptation to promote gaseous exchange, or heat loss — rather, if the grey matter is compact in at least one dimension, it is outgoing axons that may readily escape it; once outside, they club together and form the cortical white matter. If grey and white were intermixed, the average separation of neurons would be greater, creating extra neural 'wiring'. The speed of cortical computation would suffer accordingly.

The principle of economic wiring can also be invoked to account for regional specialisation of function across the surface area of the cortex. Put simply, neurons performing similar roles need to communicate, and do so more efficiently if nearby. When microelectrodes were first used for cortical recordings, an immediate discovery was that neurons in a radial column — a column perpendicular to the plane of layering — shared similar response properties: their receptive fields might all be located on the same patch of skin, or be selective for the same orientation of a contour at a certain location in the image on the retina. Moving tangentially through the sheet (parallel with the plane of layering) the discovery was that neighbouring columns have neighbouring receptive fields — the ensemble of columns ultimately giving rise to a cortical map of the relevant sensory surface. In sensory cortex, this engenders the 'one map, one area' principle for parcelling the cortical surface into discrete areas, each of which is thought to have some nuance of functional